The Eye Lens Crystallins: A Playground for Evolution

een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens het besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag 20 april 2000,
des namiddags om 1.30 uur precies

door

PAUL JOHAN LOUIS WERTEN

geboren op 10 juli 1969
te Geffen

Promotor

Prof. Dr. W.W. de Jong, UvA

Manuscriptcommissie

Dr. N.H. Lubsen
Prof. Dr. J.H. Veerkamp

ISBN: 90-9013546-4

Printing: PrintPartners Ipskamp, Enschede

The research described in this thesis was performed at the Department of Biochemistry, Faculty of Science, University of Nijmegen, the Netherlands.

The publication of this thesis was financially supported by the Dr. Ir. van der Laar Stichting.

Voor Anita

CONTENTS

CHAPTER I

General Introduction

1.1 Evolution of the eye

Since the beginnings of biological evolution, some 3.5 billion years ago, light and the day/night cycle have been one of the most influential selective forces to have ever acted on biological organisms. Not only was the light from our sun the primary source of energy that fueled early life, it also led to one of the most remarkable events during evolution: the development of light sensory organs and eyes. Even Darwin was clearly impressed by the miracle of vision, describing eyes as "organs of extreme perfection and complication". He further wrote: "that the eye … could have been formed by natural selection seems, I freely confess, absurd in the highest possible degree" [Darwin, 1859]. Even today, with our expanded knowledge, some of the awe surrounding the evolution of the eye remains.

Eyes are the primary source of sensory information for the brain in most species. Reflecting the importance of vision to animals is morphological evidence that eyes have evolved independently in very different organisms at least 40 times and perhaps as many as 65 times during evolution [Salvini-Plawen & Mayr, 1977]. Image-forming eyes arose in six of the 33 extant metazoan phyla, which contribute to about 96% of the known species that are alive today [Land & Fernald, 1992]. Indeed, paired eyes in the three major phyla (vertebrates, arthropods and mollusks) have long been considered to be the classic example of evolutionary convergence. In a way this must certainly be true, since these eyes have arisen from different tissues and immensely different solutions have been used to solve the problem of collecting and focusing light. However, it has been shown recently that at the molecular level homologous proteins like the opsins (essential for catching photons) and *Pax-6* (which plays a key role in the development of eyes) are present in the eyes of radically diverse species [Land & Fernald, 1992; Quiring *et al.*, 1994]. How is it possible, then, that the structure and development of eyes in different species, which clearly have been shown to be polyphyletic in origin, seem to be monophyletic at the molecular level?

Visual information in the form of photons has to be captured by specialized molecules and translated to electrical signals that can be interpreted by the brain. This is accomplished by the cooperation of opsins with a visual pigment or chromophore. Upon excitation by a photon, the chromophore changes in length, which in turn activates the enzymatic properties of the opsin to which it is bound, ultimately causing a decrease in current flow across the outer segment membrane of the photoreceptor cells. A crucial point to make here is the fact that the opsins have a history that actually precedes that of the eyes [Goldsmith, 1990] (see also Figure 1). Opsins consist of seven transmembrane helices connected by short loops. The chromophore is attached covalently to the seventh transmembrane domain. This is true for all

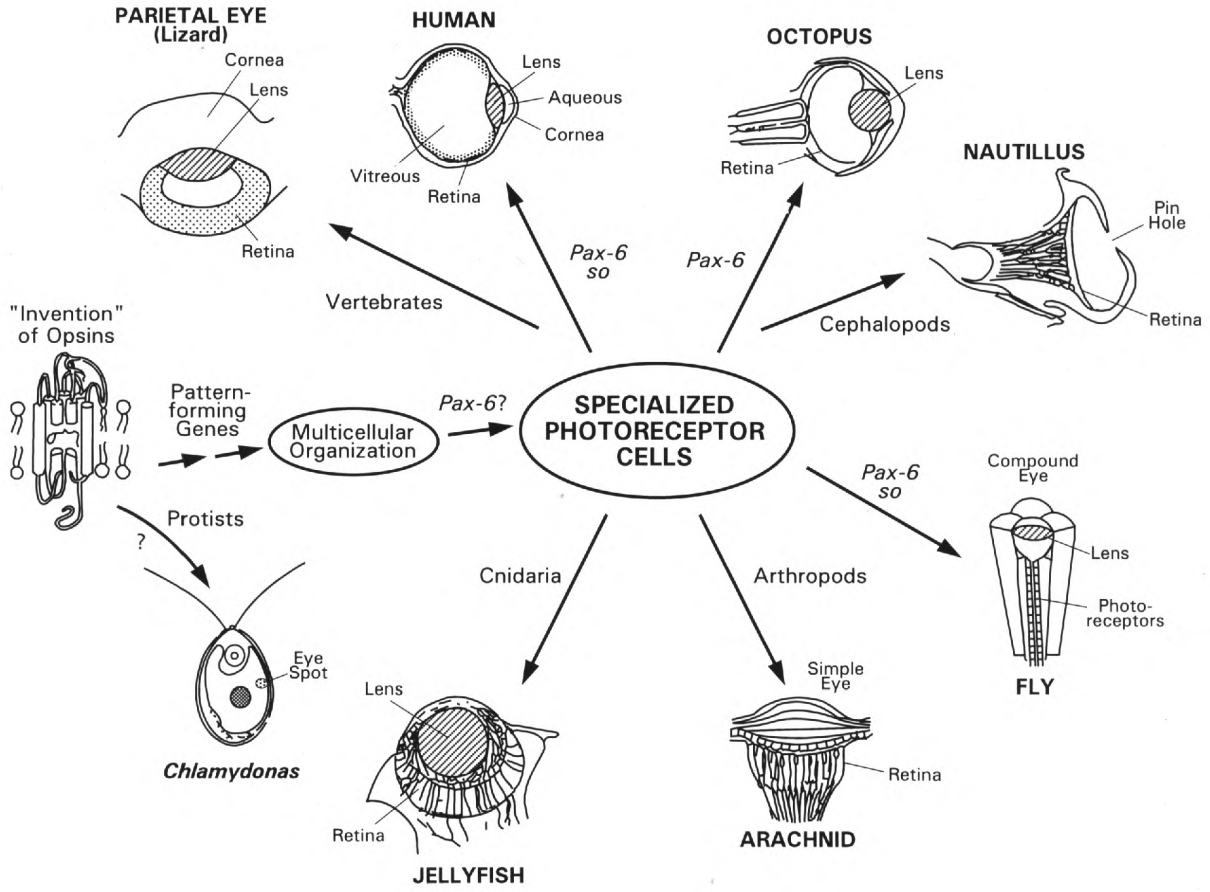


Figure 1. The evolution of eyes. Metazoan eyes may share a common origin and common molecular mechanisms of development. The 'invention' of the ancestral opsins may be an even older event. Figure taken from Wistow (1995).

metazoan opsins, and sequence analysis suggests a common ancestor for these proteins [Land & Fernald, 1992]. It is very well possible that the history of the opsins goes even further back to the archaebacterial bacteriorhodopsins. Although there are no apparent sequence similarities with the metazoan opsins, their structure and function is strikingly similar. These similarities between the bacteriorhodopsin and opsin families could be the result of common descent from a single 'invention' of this protein motif in a very early stage in evolution. Alternatively, this could mean that the two families, although very similar in structure, just converged to the same structure, since the seven-transmembrane motif is thought to be very common in membrane receptor proteins [Applebury, 1994; Larhammar *et al.*, 1993; Strader *et al.*, 1994].

In 1994, Quiring *et al.* have shown that the *eyeless* gene in *Drosophila* is homologous to the *Small eye* or *Pax-6* gene in mice. The absence of this gene causes the absence of eyes in the fruit fly, and reduced or absent eyes in the mouse. Moreover, the *eyeless* gene was shown to be able to induce ectopic eyes in the fruit fly, and rescue the *Small eye* phenotype in mice

[Halder *et al.*, 1995]. Similarly, the *Pax-6* gene of mice was also able to induce ectopic *Drosophila* eyes in fruit flies. This is very remarkable, because the compound eyes of flies are quite different from the chambered eyes of mice. It shows that *Pax-6* plays a key role in the development of eyes and must lie upstream of other genes in the developmental cascade necessary to form eyes. More and more members of the cascade are being discovered. *Sine oculus* is another homeodomain-encoding gene which is also essential for the development of eyes in the fruit fly, and it appears to have homologues that are expressed in mammalian eyes [Cheyette *et al.*, 1994]. Other pattern forming genes such as *Notch*, *Msx-1* and *-2*, and various *Hox* genes are also known to be expressed regionally during the development of eyes from various species [Beebe, 1994; Fortini *et al.*,1993; Monaghan *et al.*, 1991; Levine & Schechter, 1993]. The eye developmental cascade must have evolved early on in metazoan evolution (see Figure 1), giving organisms that possessed it a great advantage over others in being able to respond to light. As these species diverged over the next hundreds of millions of years, giving rise to various new species with very different forms of eyes, the ancient underlying molecular mechanisms have evidently been preserved.

1.2 The vertebrate eye and the eye lens

The vertebrate eye belongs to the category of so-called camera-type eyes (see Figure 2). It is a spherical organ with a single transparent opening to the anterior side, and a photosensitive layer called the retina that covers most of the inside of the otherwise opaque eyeball [Davson, 1990; Berman 1991]. Light passes through the cornea, the outermost transparent layer of the eye, is focused by the eye lens onto the retina, which in turn delivers the optic information to the brain via the optic nerve at the posterior end of the eye [Davson, 1990; Berman 1991; Walls, 1967]. The amount of light that passes through the transparent anterior opening of the eye is regulated by the iris, which extends from the ciliary body and can contract to cover a varying part of the eye lens. Apart from allowing light to enter the eye, the cornea obviously forms the barrier between the inside of the eye and the external environment. In terrestrial species the cornea also contributes greatly to the total refractive power of the eye. In addition, the cornea absorbs short-wave UV radiation, which would damage the sensitive interior of the eye [Davson, 1990; Berman 1991]. Behind the cornea lies the aqueous humor, a clear fluid that fills the anterior chamber and is essential in supplying both the cornea and the anterior part of the lens with the necessary nutrients and growth factors. The lens, also attached to the ciliary body, separates the aqueous humor from the vitreous humor that fills the posterior chamber of the eye. The vitreous humor is also transparent, but more gelatinous in nature. It

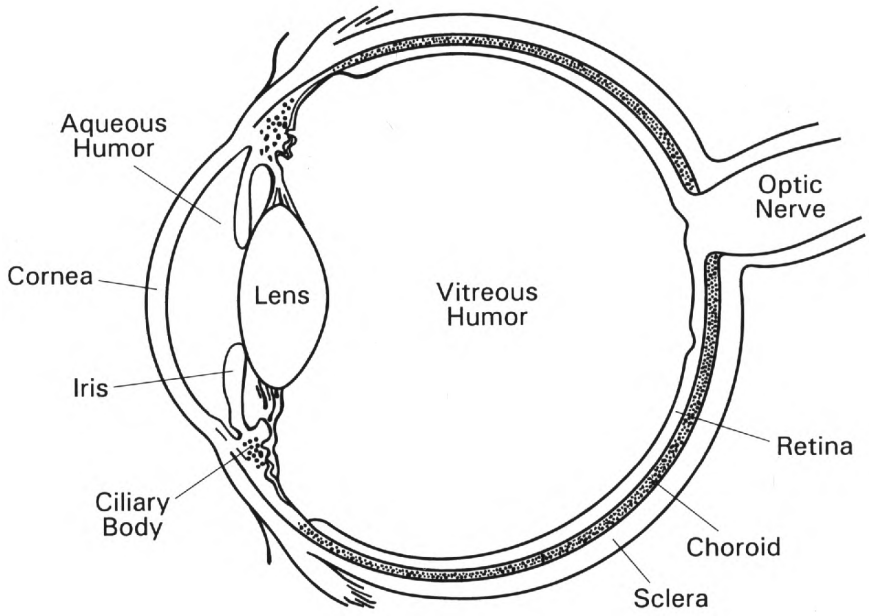


Figure 2. Structure of the human eye. Figure taken from Wistow (1995).

serves the same purpose as the aqueous humor, being a source for nutrients and growth factors [Davson, 1990; Berman 1991]. The retina consists of layers of nerve cells and the opsin containing photoreceptor cells. Interestingly, in vertebrate eyes light has to first pass through the neural layers to get to the photoreceptor cells. The photoreceptor cells function without turnover their entire life, and continuously produce membranous discs containing the opsins and other proteins of the visual cascade [Davson, 1990; Berman 1991].

An essential part of the camera-type eye is the eye lens. The eye lens is a remarkable tissue with two properties critical to vision. First, it is transparent, thus allowing light to pass through. Second, it is able to refract light, thus allowing correct focussing of light on the retina to form an image of the outside world. Responsible for both properties are the water-soluble proteins contained within the lens cells. These proteins are called 'crystallins', referring to a description of eye lenses by Celsus some 1800 years ago, who described the transparent properties of eye lenses as 'crystalloides', meaning ice-like. The high concentration of these proteins (between 20 to 60% of wet weight) [de Jong *et al.*, 1989] is responsible for the high refractive index of the lens. Transparency of the lens can be explained by the short-range order of proteins in the lens [Delaye & Tardieu, 1983], by the absence of abrupt discontinuities in refractive index, and by the few visible chromophores the lens contains. To further increase the dioptric power of the lens and to reduce spherical aberration, there is a gradual increase in refractive index from the periphery to the center of the lens [Fernald & Wright, 1983].

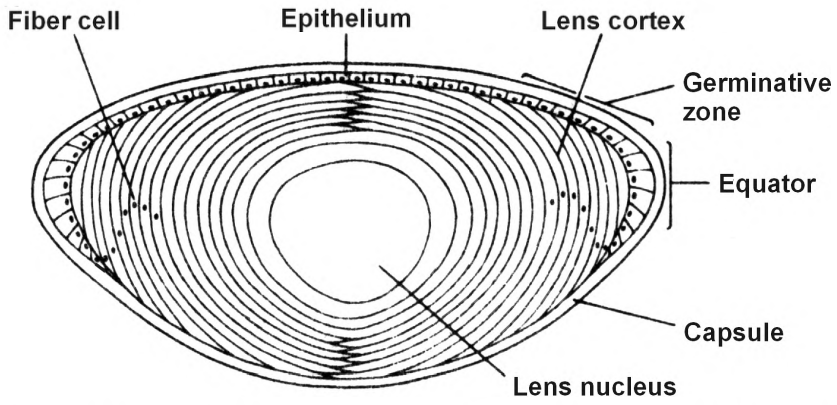


Figure 3. General structure of the vertebrate eye lens. The lens is contained in an elastic collagenous capsule and has a single layer of epithelial cells at the anterior side. These epithelial cells differentiate at the equator into extremely elongated fiber cells. Figure adapted from de Jong and Hendriks (1986).

Crucial to its functioning is the way the eye lens grows (Figure 3). Epithelial cells from the single cell layer covering the anterior side of the lens start to elongate and differentiate into elongated lens fiber cells when they reach the equatorial region, most likely by the influence of growth factors like FGF that are present in high concentration within the vitreous humor [Chamberlain & McAvoy, 1987; Chamberlain & McAvoy, 1989; Peek *et al.*, 1992; Leenders *et al.*, 1997]. By doing so, they cover previous layers of lens fiber cells. In the process they gradually lose cell organelles such as nuclei and mitochondria to reduce light diffraction [Bassnett & Beebe, 1992]. Side-by-side packing and spiraling of lens fiber cells gives rise to shells, that form the concentric layers of the eye lens, much like the layers of an onion. New layers grow throughout life, so that the fetal eye lens forms the center of the adult eye lens. Crystallin synthesis can only occur in the cortical region, the outermost layers of the lens, where the cell nuclei are still present. Since there is no protein turnover, this means that the proteins in the center of the eye lens, the nucleus, are as old as the individual organism itself. It also means that the protein composition of the various layers of the eye lens reflects the specific protein expression at that stage of development [Piatigorsky, 1981]. This gradual change in protein composition in combination with post-translational modifications of these proteins may contribute to a gradually tighter packing of the crystallins toward the center of the lens, thus forming the protein concentration gradient that is so crucial for the correct optical properties of the lens [Kenworthy *et al.*, 1994; Vérétout & Tardieu, 1989; Voorter *et al.*, 1990]. In fact, it has been theorized that the demonstrated difference in colloid osmotic pressure between proteins of the cortex and nucleus can explain about 25 percent of this protein concentration gradient [Vérétout & Tardieu, 1989]. A concentration gradient of ions

or small solutes in the opposite direction is required to completely explain the actually existing protein concentration gradient. In this respect, it is interesting to mention that such gradients indeed exist. Na^+ concentrations in the nucleus have been shown to be lower than in the cortical regions [Amoore *et al.*, 1959; Garner *et al.*, 1986]. Taurine, one of the major free amino acids in the lens, has also been shown to decrease in concentration from cortex to nucleus [Gupta & Mathur, 1983]. The early decline in total lens taurine has furthermore been implicated to be responsible for the general dehydration of the lens such as it occurs in rats between 30 and 90 days of age [Baskin *et al.*, 1977].

1.3 The crystallins

As mentioned above, there is no protein turnover in the eye lens so that the crystallins must last a lifetime. This is quite unique, since in almost all other tissues there is a steady turnover and renewal of proteins. Combined with the necessity of a very high protein concentration in the lens, this means that all crystallins must be highly water-soluble, long-living and stable proteins, resistant to deleterious influences such as radiant light, radicals and heat [de Jong *et al.*, 1989]. These requirements must certainly have imposed a strong selective force on the proteins that were to become crystallins. Nevertheless, despite these requirements the different crystallin families comprise proteins that vary greatly in size, subunit composition and physicochemical properties [de Jong *et al.*, 1989].

Two major categories of crystallins can be distinguished: the ubiquitous crystallins, those that are found in all vertebrates, and the taxon specific crystallins, those that are only found in specific groups or species [Wistow, 1993]. Belonging to the first category are the α-, β- and γ-crystallins, some of which were first discovered almost a century ago. As mentioned before, these proteins occur in all vertebrate eye lenses, although their relative amounts vary. The ubiquitous crystallins were recruited in the very beginning of lens evolution and have been maintained until the current day in lenses of widely varying accommodative and refractive power, showing their persistent evolutionary success [de Jong *et al.*, 1989; Wistow, 1993]. Belonging to category of the taxon-specific crystallins is a whole range of proteins, primarily consisting of enzymes. These proteins were most likely recruited as crystallins later on in the evolution and possibly fulfill specific needs in particular species. Some of them may be necessary to change the properties of the lens, so that it meets the requirements of the environment of that species.

1.4 The ubiquitous crystallins

α-Crystallin is a multimer of 20 kDa αA and αB subunits, forming large complexes with a molecular mass around 800 kDa [de Jong, 1981]. The present data suggest that α-crystallin originated as an abundant lens protein by increased expression of the already existing αB gene, while αA probably arose as a lens specific protein after duplication of the αB gene. Both αA and αB are part of the small heat-shock protein family [de Jong *et al.*, 1991; Wistow & Piatigorsky, 1988]. Indeed, αB is found as a heat-shock protein in tissues like kidney, heart, muscle and brain, and can be upregulated in case of stress to aid the survival of stressed cells [Aoyama *et al.*, 1993; Dasgupta *et al.*, 1992; Klemenz *et al.*, 1991]. α-Crystallin, and the homo-oligomers αA and αB, all demonstrate so-called chaperone-like properties, meaning that they can bind partially unfolded proteins *in vitro*, preventing further unfolding and aggregation of these proteins [Horwitz, 1992; Merck *et al.*, 1993; Van Boekel *et al.*, 1996]. It is not clear whether the chaperone-like properties are directly related to the protective effect these proteins have on cells. It is however certainly understandable that both of these properties could be beneficial to the lens, since lens fiber cells are constantly exposed to UV and radical stress, and damaged lens proteins could be bound by α-crystallin to prevent aggregation and loss of transparency.

The β- and γ-crystallins form a protein super-family of their own [Driessen *et al.*, 1981; Lubsen *et al.*, 1988]. Both β- and γ-crystallins consist of four so-called "Greek Key" motifs, a distinctive beta-sheet folding, arranged in two globular domains and separated by a short connecting peptide [Blundell *et al.*, 1981] (see Figure 4). The β- and γ-crystallins are thought to have evolved from a one-domain, one-motif ancestral protein [Lubsen *et al.*, 1988] (see Figure 5). After gene duplication and fusion that protein became a one-domain, two-motif protein. From there the paths of β- and γ-crystallin split up: the ancestor for the γ-crystallins lost the intron sequence between the two motifs, whereas it was retained in the ancestor of the β-crystallins. A second gene duplication and fusion event occurred in both the β- and γ-crystallin lineage, leading to two-domain, four-motif proteins closely resembling the current day β- and γ-crystallins. Evidence for this scenario comes from sequence analysis of the β- and γ-crystallin motifs [Lubsen *et al.*, 1988]. Both in β- and γ-crystallins the first and third motif are more similar to each other than to the second and fourth motif. Likewise, the second and fourth motif are more similar to each other than to the first and third motif. This clearly suggests two consecutive gene duplication and fusion events. The absence of the intron between the first and second and the third and fourth motif of the current day γ-crystallins is

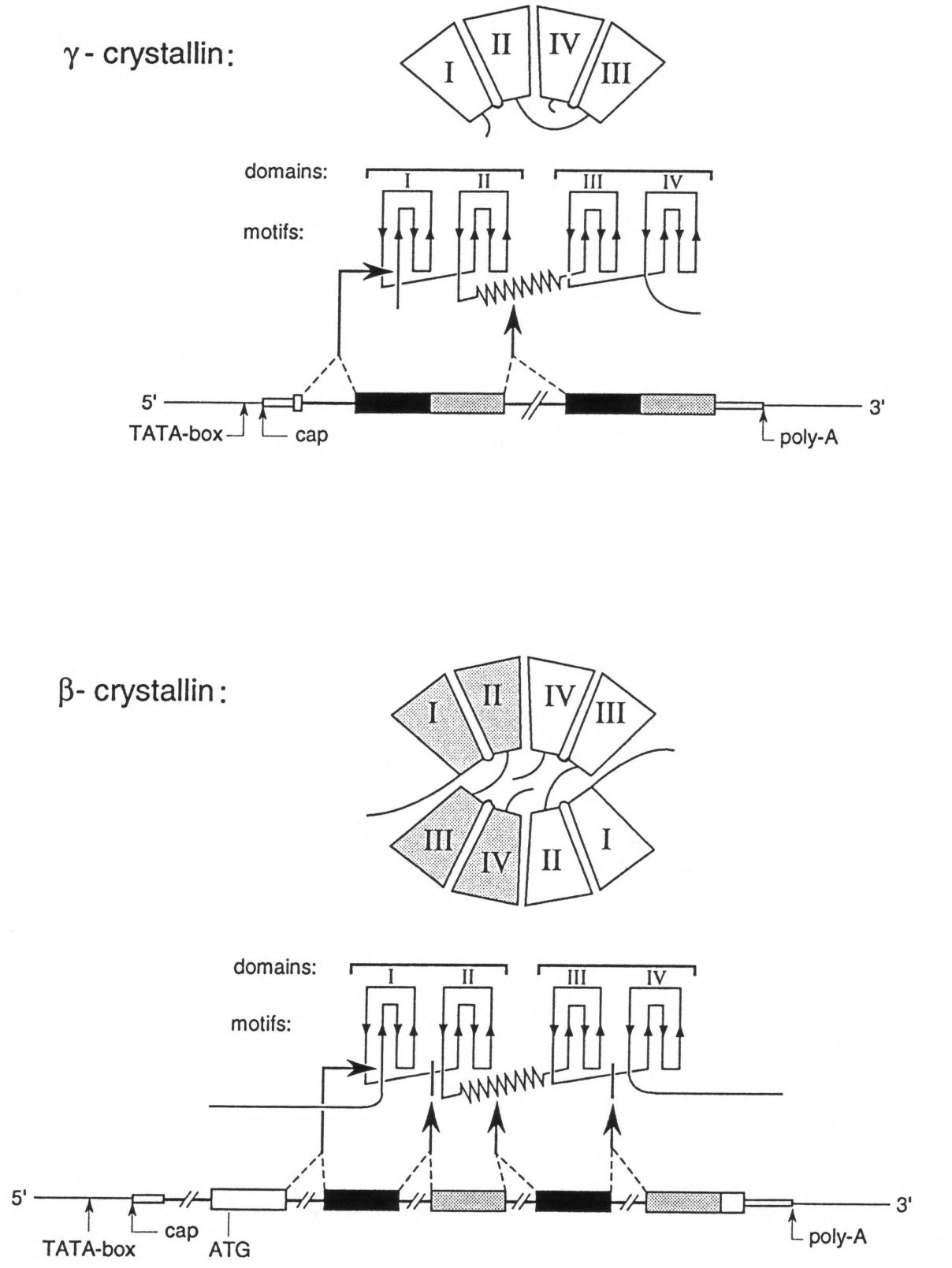


Figure 4. Schematic view of the protein and gene organization of the β- and γ-crystallins. The bottom line represents the gene organization with the exons marked by boxes; narrow boxes represent non-coding regions, wider boxes represent coding regions. Shading indicates the internal homology within the coding regions. The Greek key motifs are indicated above their coding regions. Arrows indicate the sites of interruption by introns. The tertiary structure of the proteins is schematically represented on top. Figure taken from de Jong *et al.* (1994).

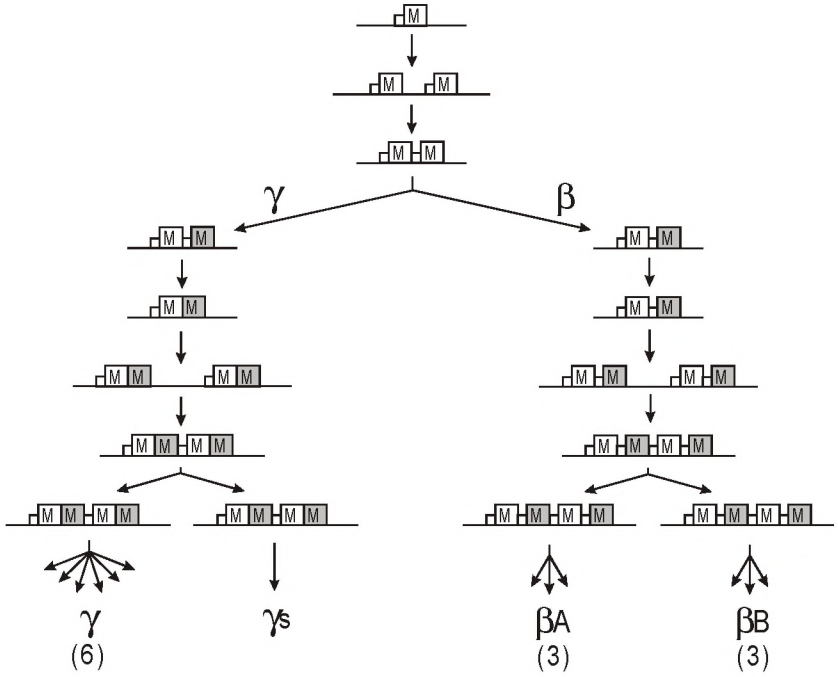


Figure 5. Schematic view of the evolution of the present day β- and γ-crystallin genes from an ancestral one motif gene. The coding region of a single motif is shown as a boxed M. White boxes and shaded boxes indicate sequence similarities between these motifs. The numbers between brackets at the bottom of the figure indicate the number of members of a particular gene family as found in mammals. Figure adapted from Lubsen *et al.*, 1988.

more likely explained by a single loss of the intron in the one-domain, two motif ancestor protein before the last duplication event, than by a loss of two introns on separate occasions in the history of the γ-crystallins. The ancestral β-crystallin gave rise to two groups of proteins, the acidic and basic β-crystallins, that now consist of 4 and 3 proteins, respectively [Berbers *et al.*, 1984]. In mammals the γ-crystallin ancestor gave rise to γS on the one hand and the more closely related γA through γF on the other hand.

Whereas the interaction between the N-terminal and C-terminal domains are intra-molecular in the γ-crystallins that therefore only occur as monomers, they are inter-molecular in the β-crystallins that obligatorily form oligomers ranging from dimers to octamers [Slingsby *et al.*, 1997]. Interestingly, X-ray crystallography has shown that the three-dimensional structure of either the upper or lower half of a β-crystallin dimer is almost identical to a γ-crystallin monomer (see Figure 6.) [Bax *et al*, 1990; Lapatto *et al.*, 1991]. Some have suggested that the longer and elongated connecting peptide of the β-crystallins is responsible for the clear difference in oligomerization behavior between the β- and the γ-crystallins [Lapatto *et al.*, 1991; Trinkl *et al.*, 1994]. Others have suggested that the fact that

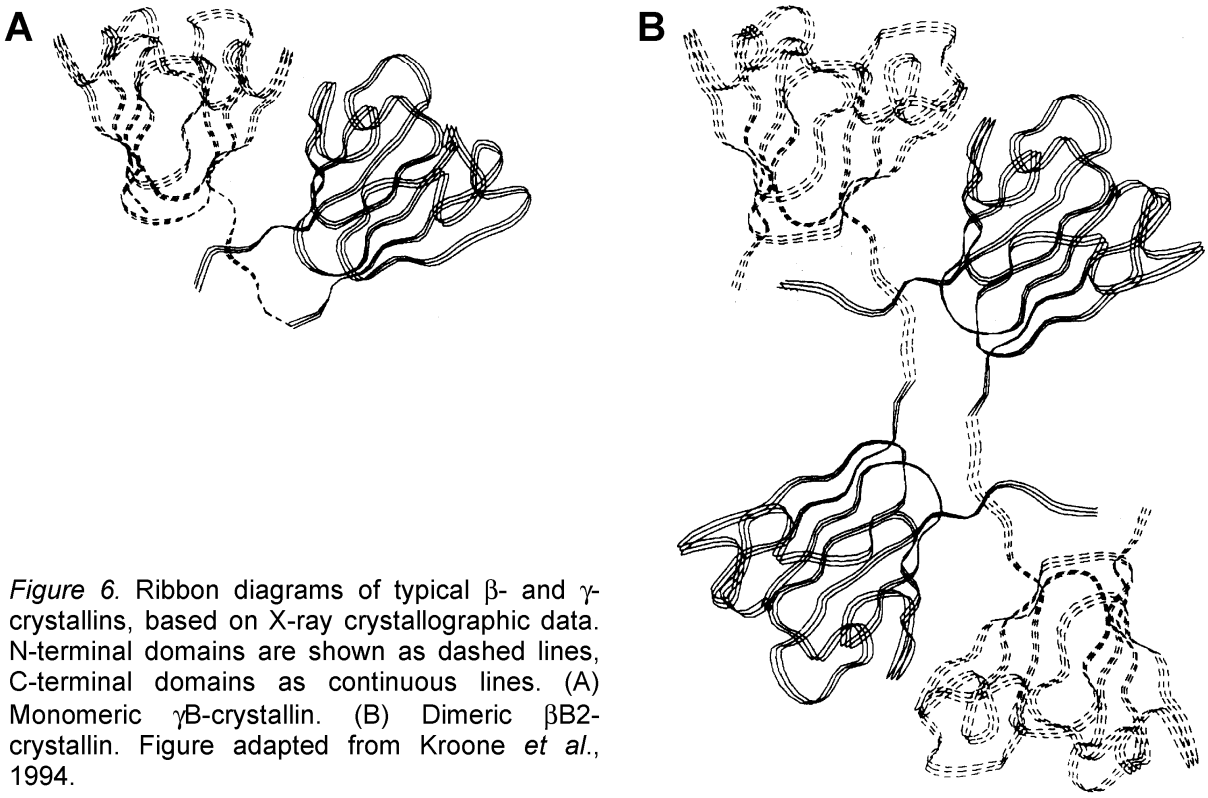


Figure 6. Ribbon diagrams of typical β- and γ-crystallins, based on X-ray crystallographic data. N-terminal domains are shown as dashed lines, C-terminal domains as continuous lines. (A) Monomeric γB-crystallin. (B) Dimeric βB2-crystallin. Figure adapted from Kroone *et al.*, 1994.

the β-crystallins have N- and sometimes C-terminal extensions, which are absent in the γ-crystallins, is responsible for this difference in oligomerization behavior [Berbers *et al.*, 1983; Hope *et al.*, 1994; Sergeev *et al.*, 1997].

Very little is known about the origin of the β- and γ-crystallins, and they are classified as structural lens proteins simply due to lack of a better understanding of the function these crystallins might have had, or still may have in other tissues. Clues seem to come from other proteins that have been found to also contain Greek key motifs. The spore coat protein S of *Myxococcus xanthus*, for instance, is a two domain/four motif protein [Wistow *et al.*, 1985]. Spherulin 3A, an encystment protein of the slime mold *Physarum polycephalum* is a one domain/two motif protein [Wistow, 1990]. Both of these proteins suggest a stress connection for the β- and γ-crystallin superfamily, since they are expressed in these organisms during unfavorable conditions. Of course it could also be supposed that the Greek key folding, which appears to be a very stable folding, is simply beneficial to all kinds of structural proteins that need to last under harsh conditions, be they dormancy proteins or lens proteins. Two other proteins recently discovered, Aim1 and EDSP/ep37, seem to point in another direction. Aim1 is a protein that contains twelve Greek key motifs arranged in six β/γ-like domains and has been found to be a tumor suppressor gene in human malignant melanoma, probably exerting

its influence via the cytoskeleton [Ray *et al.*, 1997]. EDSP/ep37 of the amphibian *Cynops pyrrhogaster* is expressed in differentiating epidermis and shows an intracellular localization consistent with an association with cytoskeleton [Wistow *et al.*, 1995]. Both of these proteins suggest a possible role for the β/γ-crystallin superfamily in cell morphology via interaction with the cytoskeleton. Interestingly, the cytoskeleton is also crucial for the structure of the elongated lens fiber cells, that undergo large changes in cytoskeletal architecture and composition during differentiation [Wistow, 1993; Wistow, 1995]. The finding that some β- and γ-crystallins can be detected in non-lens tissues during certain stages of embryonic development [Smolich *et al.*, 1994; Dirks *et al.*, 1998] may suggest a similar function.

1.5 The taxon-specific crystallins and crystallin recruitment

More than ten taxon-specific crystallins have been identified over the past years. A summary of these crystallins and their origin is given in Table I. All but one of these taxon-specific crystallins are related or identical to enzymes. Interestingly, most of these enzymes are

Table I. Occurrence and identification of taxon-specific crystallins

Crystallin	Occurrence	Relationship	References
δ	birds and reptiles	δ2 = argininosuccinate lyase	Piatigorsky *et al.*, 1988
ε	many birds and crocodiles	= lactate dehydrogenase B4	Wistow *et al.*, 1987; Hendriks *et al.*, 1988
λ	rabbits and hares	hydroxyacyl-CoA dehydrogenase	Mulders *et al.*, 1988
ι	various diurnal geckos	= cellular retinol-binding protein I	Röll *et al.*, 1996
π	diurnal geckos (genus *Phelsuma*)	= glyceraldehyde 3-phosphate dehydrogenase	Jimenez-Asensio *et al.*, 1995
τ	lampreys, some fish, birds and reptiles	= α-enolase	Wistow *et al.*, 1988
ζ	guinea pigs, camels	alcohol dehydrogenase	Huang *et al.*, 1987; Rodokanaki *et al.*, 1989
ρ	frogs (genus *Rana*)	aldose and aldehyde reductase, prostaglandin F-synthetase	Gause Jr. *et al.*, 1985; Carper *et al.*, 1987; Watanabe *et al.*, 1988
ρB	diurnal geckos (genus *Lepidodactylus*)	aldose reductase	Röll *et al.*, 1995
η	elephant shrews	= retinaldehyde dehydrogenase	Graham *et al.*, 1996
ξ	camels	quinone oxidoreductase	Duhaiman & Rabbani, 1996; Graw, 1997
μ	diurnal marsupials	ornithine cyclodeaminase	Kim *et al.*, 1992

involved in osmotic stress, UV- or oxidative stress, chemical detoxification, or interactions with the cytoskeleton. So here again there appears to be a link between stress and the recruitment of proteins as eye lens crystallins. This may indicate that the eye lens, which lacks vascularization and is continuously exposed to UV radiation and heat, can be considered a stressed tissue. The selection of proteins that could contribute to the long-term stability of the lens when expressed in high levels would therefore make good sense. Especially for the taxon-specific crystallins that have been recruited much later in evolution than the ubiquitous crystallins, properties that could protect the lens may have been an important factor in their continued use as lens proteins. Enzymes may also have been easy targets for quick selection, because a lot of these proteins were already present at low housekeeping levels in the eye lens, and may already have had promotors that could easily be modified to achieve high expression in the lens. All that seems to be necessary for minimal lens promotor activity is the presence of a TATA-box [Cvekl & Piatigorsky, 1996], which is present in most crystallins today. Binding sites for *Pax-6* or some other transcription factors that are highly expressed in the lens, such as the *Sox* proteins and the recently discovered *maf* proteins [Ogino & Yasuda, 1998; Sharon-Friling *et al.*, 1998; Kim *et al.*, 1999], and more ubiquitous transcription factors plus brain repressor elements seem to complete the picture [Wistow, 1995]. *Pax-6* plays a key role in the temporal and spatial expression pattern of crystallins in the developing eye lens. It appears to enhance expression of some crystallins [Cvekl *et al.*, 1994; Cvekl *et al.*, 1995a; Cvekl *et al.*, 1995b; Richardson *et al.*, 1995; Cvekl & Piatigorsky, 1996; Gopal-Srivastava *et al.*, 1996; Sharon-Friling *et al.*, 1998], but seems to repress expression of others [Duncan *et al.*, 1998; Haynes 2nd *et al.*, 1996].

1.6 βA3/A1-crystallin

The most intriguing members of the β-crystallin family are no doubt βA3- and βA1-crystallin. These two proteins are coded by the same βA3/A1 gene and mRNA. The two proteins are the result of a process that is called 'leaky ribosomal scanning', in which the first start codon is occasionally skipped and translation initiation can also start at the second or later AUG [Kozak, 1987; Kozak, 1991]. Leaky ribosomal scanning can occur when the first start codon lies in a 'weak' context or when the 5' UTR of the messenger is short [Kozak, 1989]. On the βA3/A1 messenger two start codons are present at the 5' end. Since these two codons lie in the same reading frame, the two proteins are identical, except for the fact that the N-terminal extension of βA1 is 17 amino acids shorter than the N-terminal extension of βA3. It is not clear whether leaky ribosomal scanning of the βA3/A1 messenger occurs because of the

unfavorable context of the first start codon in most animals, or because of the short 5' UTR of this messenger, which is only 5 nucleotides long in most species [Gorin & Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Peterson & Piatigorsky, 1986] and has been reported to be 7 nucleotides in humans [Hogg *et al.*, 1986]. In Chapter IV the process of leaky ribosomal scanning of the βA3/A1 messenger is therefore studied.

Of all β-crystallins, the globular domains of βA3 and βA1 have been most conserved [Aarts *et al.*, 1989]. In contrast, the N-terminal extensions are the least conserved among the β-crystallin extensions. Interestingly, however, the length of the N-terminal extensions of βA3 and βA1 -- 30 and 17 amino acid residues respectively -- and the mechanism of leaky ribosomal scanning have been perfectly conserved during at least 350 million years of evolution, since βA3 and βA1 can be found in species as diverse as chicken and human [Gorin & Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Peterson & Piatigorsky, 1986; Hogg *et al.*, 1986], and perhaps in frogs as well [Lu *et al.*, 1996]. Although there are numerous examples of leaky ribosomal scanning in viral genes, only few are known in eukaryotic genes. In the cases that are known, the use of this mechanism had functional implications for the proteins involved [Shaper *et al.*, 1988; Tuboi *et al.*, 1990; Descombes & Schibler, 1991; Slusher *et al.*, 1991; Pietrini *et al.*, 1992; Lin *et al.*, 1993; Ossipow *et al.*, 1993; Tenhunen & Ulmanen, 1993; Spotts *et al.*, 1997]. Together with the fact that the arms of the β-crystallins have been suggested to be involved in the oligomerization of the β-crystallins [Berbers *et al.*, 1983; Hope *et al.*, 1994; Sergeev *et al.*, 1997], investigating the oligomerization behavior of these two crystallins may not only provide information as to why both of these proteins and the unusual mechanism by which they are produced have been maintained so long during evolution, it may also shed some light on the oligomerization of β-crystallins in general. Chapter II compares the oligomerization behavior of βA3 and βA1, both as purified recombinant proteins and as part of bovine β-crystallin fractions. In Chapter V the specific oligomerization of βA3 with βB2 -- another major β-crystallin subunit -- is studied in more detail.

During maturation of the eye lens, all crystallins are subject to post-translational modifications. It has been suggested that the truncation of the β-crystallin extensions due to protease activity can cause insolubilization of these crystallins and may lead to opacification of the eye lens and thus to cataract [Pierscionek & Augusteyn, 1988; Harding, 1991; David *et al.*, 1994]. To gain insight into this phenomenon, we identified truncation and modification products of βA3 and βA1 in the bovine lens. Water-soluble and -insoluble fractions of nucleus and cortex from bovine lenses were compared with increasing age. The results and the implications for the optical quality of the eye lens are discussed in Chapter III.

1.7 Iota-crystallin

Only very recently the first non-enzyme taxon-specific crystallin was discovered in the eye lenses of the diurnal gecko *Lygodactylus picturatus* [Röll *et al.*, 1996]. The protein, a monomer of 16 kDa, was given the name iota-crystallin because of its small size (iota is the smallest letter of the Greek alphabet). A partial N-terminal protein sequence of iota-crystallin showed greatest homology with the rat cellular retinol-binding protein type I (CRBP I). In contrast to CRBP I that has retinol as its natural ligand, iota-crystallin was found to contain *all-trans*-3,4-didehydroretinol (vitamin A_2) in the eye lenses of *L. picturatus*. It is the iota-crystallin · vitamin A_2 complex that gives the eye lenses of this gecko their dark yellow color. This color reduces chromatic aberration of the eye lens and is able to filter out short wave UV radiation, thus protecting the gecko eye from UV damage. It makes good sense for this animal to have such protection, since they live in the high ambient light environment of the African savanna [Röll, 1994], and they have no eyelids and cannot regulate the aperture of their pupil to limit the amount of light that enters their eyes.

Geckos are thought to have evolved from diurnal lizards and became strictly nocturnal animals. After adapting to their nocturnal environment, some of these geckos reverted back to being diurnal, while others maintained their nocturnal lifestyle [Walls, 1934; Walls, 1942]. These dramatic changes in their evolution must certainly have required quick changes to the eyes and lenses of these animals, and this is most likely the reason why so many taxon-specific proteins have already been discovered in these animals [Jimenez-Asensio *et al.*, 1995; Röll *et al.*, 1995; Röll *et al.*, 1996].

Since the sequence of iota-crystallin was incomplete and the sequence for the gecko CRBP I was not known, it was not clear whether iota-crystallin was identical to gecko CRBP I and had gained a second function as a lens protein, or whether it represents a gene-duplication product of gecko CRBP I that was optimized to function in the lens. This point is resolved in Chapter VI by cloning and sequencing of iota-crystallin and gecko CRBP I. In addition, a model of iota-crystallin was created to show what amino acid changes make it more suitable to function as a lens protein. The ligand-binding properties of recombinant iota-crystallin have also been determined.

REFERENCES

Aarts HJM, Jacobs EH, van Willigen G, Lubsen NH & Schoenmakers JGG (1989). *J. Mol. Evol.* **28**, 313-321.

Amoore JE, Bartley W & van Heyningen R (1959). *Biochem. J.* **72**, 126-133.

Applebury ML (1994). *Soc. Gen. Physiol. Ser.* **49**, 235-248.

Aoyama A, Fröhli E, Schäfer R & Klemenz R (1993). *Mol. Cell. Biol.* **13**, 1824-1835.

Baskin SI, Cohn EM & Kocsis JJ (1977). *Exp. Eye Res.* **24**, 315-319.

Bassnett S & Beebe DC (1992). *Dev. Dyn.* **194**, 85-93.

Bax B, Lapatto R, Nalini V, Driessen H, Lindley PF, Mahadevan D, Blundell TL & Slingsby C (1990). *Nature* **347**, 776-780.

Beebe DC (1994). *Invest. Ophthalmol. Vis. Sci.* **35**, 2897-2900.

Berbers GA, Brans AM, Hoekman WA, Slingsby C, Bloemendal H & de Jong WW (1983). *Biochim. Biophys. Acta* **748**, 213-219.

Berbers GA, Hoekman WA, Bloemendal H, de Jong WW, Kleinschmidt T & Braunitzer G (1984). *Eur. J. Biochem.* **139**, 467-479.

Berman ER (1991). *Biochemistry of the eye* (1st ed.), Plenum Press, New York.

Blundell T, Lindley P, Miller L, Moss D, Slingsby C, Tickle I, Turnell B & Wistow G (1981). *Nature* **289**, 771-777.

Carper D, Nishimura C, Shinohara T, Dietzchold B, Wistow G, Craft C, Kador P & Kinoshita JH (1987). *FEBS Letters* **220**, 209-213.

Chamberlain CG & McAvoy JW (1987). *Curr. Eye Res.* **6**, 1165-1168.

Chamberlain CG & McAvoy JW (1989). *Growth Factors* **1**, 125-134.

Cheyette BN, Green PJ, Martin K, Garren H, Hartenstein V & Zipurski SL (1994). *Neuron* **12**, 977-996.

Cvekl A, Sax CM, Bresnick EH & Piatigorsky J (1994). *Mol. Cell. Biol.* **14**, 7363-7367.

Cvekl A, Kashanchi F, Sax CM, Bradly J & Piatigorsky J (1995a). *Mol. Cell. Biol.* **15**, 653-660.

Cvekl A, Sax CM, Li X, MacDermott JB & Piatigorsky J (1995b). *Proc. Natl. Acad. Sci. USA* **92**, 4681-4685.

Cvekl A & Piatigorsky J (1996). *Bioessays* **18**, 621-630.

Darwin C (1859). *On the origin of the species.* Murray, London.

Dasgupta S, Hohman TC & Carper D (1992). *Exp. Eye Res.* **54**, 461-470.

David LL, Azuma M & Shearer TR (1994). *Invest. Ophthalmol. Vis. Sci.* **35**, 785-793.

Davson H (1990). *Physiology of the eye* (5th ed.), The Macmillan Press Ltd., London.

de Jong WW (1981). In *Molecular and cellular biology of the eye lens.* (H. Bloemendal ed.), pp. 221-278, John Wiley and Sons.

de Jong WW & Hendriks W (1986). *J. Mol. Evol.* **24**, 121-129.

de Jong WW, Hendriks W, Mulders JW & Bloemendal H (1989). *Trends Biochem. Sci.* **14**, 365-368.

de Jong WW, Leunissen JAM & Voorter CEM (1993). *Mol. Biol. Evol.* **10**, 103-126.

de Jong WW, Lubsen NH & Kraft HJ (1994). *Prog. Ret. Eye Res.* **13**, 391-442.

Delaye M & Tardieu A (1983). *Nature* **302**, 415-417.

Descombes P & Schibler U (1991). *Cell* **67**, 569-579.

Dirks RPH, van Genesen ST, Krüse J, Jorissen L & Lubsen NH (1998). *Exp. Eye Res.* **66**, 267-269.

Driessen HPC, Herbrink P, Bloemendal H & de Jong WW (1981). *Eur. J Biochem.* **121**, 83-91.

Duhaiman AS & Rabbani N (1996). *Biochem. Biophys. Res. Comm.* **221**, 229-233.

Duncan MK, Haynes JI 2nd, Cvekl A & Piatigorsky J (1998). *Mol. Cell. Biol.* **18**, 5579-5586.

Fernald RD & Wright SE (1983). *Nature* **301**, 618-620.

Fortini ME, Rebay I, Caron LA & Artavanis-Tsakonas S (1993). *Nature* **365**, 555-557.

Garner WH, Hilal SH, Lee SW & Spector A (1986). *Proc. Natl. Acad. Sci. USA* **83**, 1901-1905.

Gause GG Jr., Tomarev SI, Arutyunyan RDKG & Dolgilevich SM (1985). In *The lens: transparency and cataract* (G. Duncan ed.), pp. 171-179, Eurage.

Goldsmith TH (1990). *Q. Rev. Biol.* **65**, 281-322.

Gopal-Srivastava R, Cvekl A & Piatigorsky J (1996*J. Biol. Chem.* 271, 23029-23036.

Gorin MB & Horwitz J (1984). *Curr Eye Res*, **3**, 939-948.

Graham C, Hodin J & Wistow G (1996). *J. Biol. Chem.* **271**, 15623-15628.

Graw J (1997). *Biol. Chem.* **378**, 1331-1348.

Gupta K & Mathur RL (1983). *Exp. Eye Res.* **37**, 379-384.

Halder G, Callaerts P & Gehring WJ (1995). *Curr. Opin. Gen. Dev.* **5**, 602-609.

Harding J (1991). In *Cataract Biochemistry Epidemiology and Pharmacology*, pp. 191, Chapman and Hall, London.

Haynes JI 2nd, Duncan MK & Piatigorsky J (1996). *Dev. Dyn.* **207**, 75-88.

Hendriks W, Mulders JW, Bibby MA, Slingsby C, Bloemendal H & de Jong WW (1988). *Proc. Natl. Acad. Sci. USA* **85**, 7114-7118.

Hogg D, Tsui LC, Gorin M & Breitman ML (1986). *J. Biol. Chem.* **261**, 12420-12427.

Hope JN, Chen HC & Hejtmancik JF (1994). *Protein Eng.* **7**, 445-451.

Horwitz J (1992). *Proc. Natl. Acad. Sci. USA* **89**, 10449-10453.

Huang QL, Russell P, Stone SH & Zigler JS Jr. (1987) *Curr. Eye Res.* **6**, 725-732.

Jimenez-Asensio J, Gonzalez P, Zigler JS Jr. & Garland DL (1995). *Biochem. Biophys. Res. Comm.* **209**, 796-802.

Kenworthy AK, Magid AD, Oliver TN & McIntosh TJ (1994). *Exp. Eye Res.* **59**, 11-30.

Kim JI, Li T, Ho IC, Grusby MJ & Glimcher LH (1998). *Proc. Natl. Acad. Sci. USA* **96**, 3781-3785.

Kim RY, Gasser R & Wistow GJ (1992). *Proc. Natl. Acad. Sci. USA* **89**, 9292-9296.

Klemenz R, Fröhli E, Steiger RH, Schäfer R & Aoyama A (1991). *Proc. Natl. Acad. Sci. USA* **88**, 3652-3656.

Kozak M (1987). *Nucleic Acids Res.* **15**, 8125-8148.

Kozak M (1989). *J. Cell. Biol.* **108**, 229-241.

Kozak M (1991). *J. Cell. Biol.* **115**, 887-903.

Land MF & Fernald RD (1992). *Annu. Rev. Neurosci.* **15**, 1-29.

Lapatto R, Nalini V, Bax B, Driessen H, Lindley PF, Blundell TL & Slingsby C (1991). *J. Mol. Biol.* **222**, 1067-1083.

Larhammar D, Blomqvist AG & Wahlestedt C (1993). *Drug Des. Discov.* **9**, 179-188.

Leenders WPJ, van Genesen ST, Schoenmakers JGG, van Zoelen EJJ & Lubsen NH (1997). *Mech. Devel.* **67**, 193-201.

Levine EM & Schechter N (1993). *Proc. Natl. Acad. Sci. USA* **90**, 2729-2733.

Lin FT, MacDougald OA, Diehl AM & Lane MD (1993). *Proc. Natl. Acad. Sci. USA* **90**, 9606-9610.

Lu SF, Pan FM & Chiou SH (1996). *Biochem. Biophys. Res. Commun.* **221**, 219-228.

Lubsen NH, Aarts HJM & Schoenmakers JGG (1988). *Prog. Biophys. Mol. Biol.* **51**, 47-76.

Merck KB, Groenen PJ, Voorter CF, de Haard-Hoekman WA, Horwitz J, Bloemendal H & de Jong WW (1993). *J. Biol. Chem.* **268**, 1046-1052.

Monaghan AP, Davidson DR, Sime C, Graham E, Baldock R, Bhattacharya SS & Hill RE (1991). *Development* **112**, 1053-1061.

Mulders JW, Hendriks W, Blankesteijn WM, Bloemendal H & de Jong WW (1988). *J. Biol. Chem.* **263**, 15462-15466.

Ossipow V, Descombes P & Schibler U (1993). *Proc. Natl. Acad. Sci. USA* **90**, 8219-8223.

Ogino H & Yasuda K (1999). *Science* **280**, 115-118.

Peek R, McAvoy JW, Lubsen NH & Schoenmakers JGG (1992). *Dev. Biol.* **152**, 152-160.

Peterson CA & Piatigorsky J (1986). *Gene* **45**, 139-147.

Piatigorsky J (1981). *Differentiation* **19**, 134-153.

Piatigorsky J, O'Brien WE, Norman BL, Kalumuck K, Wistow GJ, Borras T, Nickerson JM & Wawrousek EF (1988). *Proc. Natl. Acad. Sci. USA* **85**, 3479-3483.

Pierscionek B & Augusteyn RC (1988). *Curr. Eye Res.* **7**, 11-23.

Pietrini G, Aggujaro D, Carrera P, Malyszko J, Vitale A & Borgese N (1992). *J. Cell Biol.* **117**, 975-986.

Quax-Jeuken Y, Janssen C, Quax W, van den Heuvel R & Bloemendal H (1984). *J. Mol. Biol.* **180**, 457-472.

Quiring R, Walldorf U, Kloter U & Gehring WJ (1994). *Science* **265**, 785-789.

Ray ME, Wistow G, Su YA, Meltzer PS & Trent JM (1997). *Proc. Natl. Acad. Sci. USA* **94**, 3229-3234.

Richardson J, Cvekl A & Wistow G (1995). *Proc. Natl. Acad. Sci. USA* **92**, 4676-4680.

Rodokanaki A, Holmes RK & Borras T (1989). *Gene* **78**, 215-224.

Röll B (1994). *Sauria* **16S**, 307-310.

Röll B, van Boekel MA, Amons R & de Jong WW (1995). *Biochem. Biophys. Res. Comm.* **217**, 452-458.

Röll B, Amons R & de Jong WW (1996). *J. Biol. Chem.* **271**, 10437-10440.

Salvini-Plawen LV & Mayr E (1977). *Evol. Biol.* **10**, 207-263.

Sharon-Friling R, Richardson J, Sperbeck S, Lee D, Rauchman M, Maas R, Swaroop A & Wistow G (1998). *Mol. Cell. Biol.* **18**, 2067-2076.

Sergeev Y, Wingfield P, Hope J & Hejtmancik JF (1997). *Invest. Ophtalmol. Vis. Sci.* **38**, S206.

Shaper NL, Hollis GF, Douglas JG, Kirsch IR & Shaper JH (1988). *J. Biol. Chem.* **263**, 10420-10428.

Slingsby C, Norledge B, Simpson A, Bateman OA, Wright G, Driessen HPC, Lindley PF, Moss DS & Bax B (1997). *Prog. Ret. Eye Res.* **16**, 3-29.

Slusher LB, Gillman EC, Martin NC & Hopper AK (1991). *Proc. Natl. Acad. Sci.* USA **88**, 9789-9793.

Smolich BD, Tarkington SK, Saha MS & Grainger RM (1994). *Mol. Cell. Biol.* **14**, 1355-1363.

Spotts GD, Patel SV, Xiao Q & Hann SR (1997). *Mol. Cell. Biol.* **17**, 1459-1468.

Strader CD, Fong TM, Tota MR, Underwood D & Dixon RA (1994). *Annu. Rev. Biochem.* **63**, 101-132.

Trinkl S, Glockshuber R & Jaenicke R (1994). *Protein Sci.* **3**, 1392-1400.

Tuboi S, Suzuki T, Sato M & Yoshida T (1990). *Adv. Enzyme Regul.* **30**, 289-304.

Tenhunen J & Ulmanen I (1993). *Biochem. J.* **296**, 595-600.

van Boekel MA, Hoogakker SE, Harding JJ & de Jong WW (1996). *Ophthalmic Res.* **28S**, 32-38.

Vérétout F & Tardieu A (1989). *Eur. Biophys. J.* **17**, 61-68.

Voorter CEM, de Haard-Hoekman WA, Hermans MMP, Bloemendal H & de Jong WW (1990). *Exp. Eye Res.* **50**, 429-437.

Walls GL (1934). *Am. J. Ophthalm.* **17**, 892-915.

Walls GL (1942). *The vertebrate eye and its adaptive radiation*, pp. 622-640, Cranbrook Institute of Science, Bloomfield Hills, MI.

Watanabe K, Fujii Y, Nakayama K, Ohkubo H, Kuramitsu S, Kagamiyama H, Nakanishi S & Hayaishi O (1988). *Proc. Natl. Acad. Sci. USA* **85**, 11-15.

Wistow G, Summers L & Blundell T (1985). *Nature* **315**, 771-773.

Wistow GJ, Mulders JW & de Jong WW (1987). *Nature* **326**, 622-624.

Wistow GJ, Lietman T, Williams LA, Stapel SO, de Jong WW, Horwitz J & Piatigorsky J (1988). *J. Cell. Biol.* **107**, 2729-2736.

Wistow G & Piatigorsky J (1988). *Ann. Rev. Biochem.* **57**, 479-504.

Wistow G (1990). *J. Mol. Evol.* **30**, 140-145.

Wistow G (1993). *Trends Biochem. Sci.* **18**, 301-306.

Wistow GJ (1995). *Molecular biology and evolution of crystallins: gene recruitment and multifunctional proteins in the eye lens*, R.G. Landes Company, Austin, TX.

Wistow G, Jaworski C & Rao PV (1995). *Exp. Eye Res.* 61, 637-639.

CHAPTER II

The elusive role of the N-terminal extension of βA3- and βA1-crystallin

Paul J.L. Werten, John A. Carver, Rainer Jaenicke & Wilfried W. de Jong

Protein Engineering **9**, 1021-1028.

2.1 Abstract

β-Crystallins are structural lens proteins with a conserved two-domain structure and variable N- and C-terminal extensions. These extensions are assumed to be involved in quaternary interactions within the β-crystallin oligomers or with other lens proteins. Therefore, the production of βA3- and βA1-crystallin from the single βA3/A1 mRNA by dual translation initiation is of interest. These crystallins are identical, except that βA1 has a much shorter N-terminal extension than βA3. This rare mechanism has been conserved for over 250 million years during the evolution of the βA3/A1 gene, suggesting that the generation of different N-terminal extensions confers a selective advantage. We therefore compared the stability and association behaviour of recombinant βA3- and βA1-crystallin. Both proteins are equally stable in urea- and pH-induced denaturation experiments. Gel filtration and analytical ultracentrifugation established that βA3 and βA1 both form homodimers. In the water-soluble proteins of bovine lens, βA3 and βA1 are present in the same molecular weight fractions, indicating that they oligomerize equally with other β-crystallins. ^{1}H-NMR spectroscopy showed that residues Met1 to Asn22 of the N-terminal extension of βA3 have great flexibility and are solvent exposed, excluding them from protein interactions in the homodimer. These results indicate that the different N-terminal extensions of βA3 and βA1 do not affect their homo- or heteromeric interactions.

2.2 Introduction

The transparency and refractive index of the vertebrate eye lens depend on, respectively, the short-range interactions and concentrations of the soluble proteins within the lens fiber cells (Benedek, 1971; Delaye and Tardieu, 1983). In mammals, three major groups of lens proteins can be distinguished: the α-, β- and γ-crystallins (Wistow and Piatigorsky, 1988). The β- and γ-crystallins belong to the same superfamily of lens proteins (Driessen *et al.*, 1981; Lubsen *et al.*, 1988). There are seven different β-crystallins, subdivided into four acidic and three basic proteins (Berbers *et al.*, 1984). The γ-crystallins also comprise seven proteins. There is approximately 30% sequence identity between the β- and γ-crystallins. These proteins all consist of four sequence-related Greek key β-sheet motifs arranged in an N-terminal and a C-terminal globular domain (Blundell *et al.*, 1981; Driessen *et al.*, 1981; Wistow *et al.*, 1983; White *et al.*, 1989; Bax *et al.*, 1990; Lapatto *et al.*, 1991). The γ-crystallins exclusively occur as monomers, whereas the β-crystallins form homo- and heteromeric dimers and higher oligomers. One of the factors that could be responsible for this difference in association

behaviour is the presence of sequence extensions in the β-crystallins, which are absent in the majority of γ-crystallins (Wistow *et al.*, 1981; Slingsby *et al.*, 1988). γS-crystallin is the exception in that it has a short, flexible N-terminal extension of four amino acids (Quax-Jeuken *et al.*, 1985; Cooper *et al.*, 1994).

With respect to their N-terminal extensions, two acidic β-crystallins, βA3 and βA1, are of particular interest. These two proteins are coded by the same gene and translated from the same mRNA by dual translation initiation as a result of 'leaky ribosomal scanning', where the first start codon is occasionally skipped and translation starts at a second start codon (Kozak, 1989, 1991). Because these start codons on the βA3/A1 mRNA are in the same reading frame, the resulting proteins are identical, except that βA1 lacks the first 17 amino acids in its N-terminal extension. This relatively rare mechanism leads to the presence of βA3- and βA1-crystallin in mammals and birds (Inana *et al.*, 1982; Gorin and Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Hogg *et al.*, 1986; Peterson and Piatigorsky, 1986), and has thus been maintained during at least 250 million years of evolution. This suggests a selective advantage for the presence of these N-terminal extensions of different lengths on the same crystallin. The comparison of βA3- and βA1-crystallin thus offers a unique opportunity for studying the possible role of the N-terminal extension in the formation of dimers and higher oligomers.

By removing the complete 30 residue N-terminal extension of βA3, Hope *et al.* (1994) obtained evidence that this extension is required for dimer formation and higher-order association. However, similar experiments with another β-crystallin, βB2, indicated that in this protein the N-terminal arm was not required for dimerization (Trinkl *et al.,* 1994; Kroone *et al.*, 1994). Consistent with these results, it has been shown by ^{1}H-NMR spectroscopy that the N- and C-terminal extensions of βB2 are of little ordered structure and flex freely from the main body of the protein (Carver *et al.*, 1993). It is therefore of interest to investigate whether and how the difference in length of the N-terminal extension of βA3 and βA1 influences their association behaviour, and to assess the flexibility of this extension.

In this paper we conclude that the difference in length of the N-terminal extension of βA3- and βA1-crystallin does not affect either their ability to form homodimers or their tendency to form higher oligomers in the presence of other crystallins in the lens. The stabilities of the two proteins are also similar. ^{1}H-NMR spectroscopy reveals that the first 22 residues of the N-terminal extension of βA3-crystallin can readily be detected, indicating that this part of the N-terminal extension is not involved in oligomerization. The functional role of the N-terminal extension of βA3 and βA1 thus remains elusive.

2.3 Materials and methods

Construction of βA3 and βA1 expression vectors

The complete sequence of βA3-crystallin was cloned into the pBluescript II sk^+ vector (pBSβA3) as described previously by Groenen *et al.* (1994). This cDNA is a chimaera coding for residues 1-47 of bovine βA3 and residues 48-215 of rat βA3. From this βA3 cDNA clone, the corresponding βA1 encoding clone was constructed. A new *Nde*I restriction site, containing the initiation codon ATG of βA1, was introduced at position 48 of the βA3 coding sequence by PCR-mediated mutagenesis, using an internal primer containing the desired mutations (TCCAACCACa**cAtATG**GCTCAAAC) (*Nde*I-site in bold, mismatches from the original sequence in lower case) and a second primer annealing to nucleotides 430-453 (CTGATGGGAAGGAATGTTCGGTAC). The resulting PCR-product, with a length of 415 bp, was then digested with *Nde*I and *Bgl*II, generating a 369 bp fragment. This fragment was cloned into the corresponding *Nde*I and *Bgl*II sites of pBSβA3, generating the complete βA1 sequence in the pBluescript II sk^+ vector (pBSβA1). The sequence of the amplified region of this cDNA was confirmed by DNA sequencing (Sequenase 2.0, Promega). The βA3-crystallin coding sequence was cloned into the pET3b vector as described by Groenen *et al.* (1994), generating the expression vector pETβA3. The βA1-crystallin coding sequence was excised from pBSβA1 as a 0.8 kb fragment by *Nde*I and *Bam*HI digestion, and ligated into the *Nde*I and *Bam*HI sites of the pET3a vector, generating the expression vector pETβA1.

Expression of βA3- and βA1-crystallin

Both expression vectors were transfected into *Escherichia coli* BL21(DE3) bacterial cells. 2YT-medium containing 100 µg/ml ampicilin was inoculated by addition of 0.5% of overnight cultures of the respective clones. After 4 h of growth at 37°C, protein expression was induced by addition of IPTG to a final concentration of 0.5 mM. Cells were harvested after 4 h by centrifugation at 5000 rpm for 15 min. For bacteria expressing βA3, the cell pellet of 500 ml culture was resuspended in 10 ml of 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA. This suspension was freeze-thawed 4 times (at -80°C and 15°C, respectively) and sonicated 3 times for 30 seconds, to disrupt the cells. A 10 ml volume of 1.5 M NaCl, 12 mM $MgCl_2$ and 20 µg *DNase*I was added and the suspension was incubated on ice for 1 h. After centrifugation at 13,000 rpm for 45 min at 4°C, the supernatant was dialysed overnight against 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, and stored at -20°C until further use. For the

isolation of βA1, a modified procedure was used. The cell pellet of 500 ml culture was resuspended in 10 ml 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 10 mM NaCl (TEN_{10}). This suspension was freeze-thawed 4 times and sonicated 3 times for 30 seconds. 10 ml of TEN_{10} was added, together with 40 µg *Dnase*I, and the suspension was incubated on ice for 2 h. After centrifugation at 13,000 rpm for 45 min at 4°C, the supernatant was stored at -20°C until further use.

Purification of recombinant protein

For the purification of βA3-crystallin, 30 ml fractions of the water soluble bacterial lysate (see above) were applied to a DEAE Sepharose Fast Flow column (40 ml). The column was eluted with 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, at a flow rate of 0.6 ml/min. Under these conditions βA3 does not bind to the column, whereas almost all *E. coli* protein remains bound. Fractions containing βA3 of sufficient purity were pooled. After dialysis against water, the protein solution was lyophilised and stored for further experiments.

In a similar way βA1-crystallin could be purified. Portions of 25 ml of water soluble bacterial lysate (see above) were applied to a DEAE Sepharose Fast Flow column (50 ml). The column was eluted with TEN_{10} buffer at a flow rate of 3.5 ml/min. Under these conditions βA1 does not bind to the column, whereas almost all *E. coli* proteins are retained. Fractions of sufficient purity from 6 runs were pooled, dialysed against water and lyophilised. The protein was stored at -20°C, until further purification could be performed. This purification was accomplished by dissolving the protein in 10 ml of 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 100 mM NaCl (TEN_{100}), and applying 1 ml portions of this solution to a Superdex 75 PG 16/60 gel filtration column (Pharmacia). The column was eluted with TEN_{100}, at a flow rate of 1 ml/min. The major peak containing βA1 was pooled from 10 runs and stored at -20°C.

N-terminal sequence analysis of recombinant proteins

The purified recombinant proteins βA3 and βA1 were checked for any proteolysis by N-terminal sequence analysis. For this purpose, 300 pmol of both proteins were applied to a 13% polyacrylamide gel. After separation, the proteins were blotted onto a PVDF membrane (Immobilon-P, Millipore). The blots were stained with Coomassie blue and the bands corresponding to βA3 and βA1 were excised and destained. Amino acid sequencing was performed at the SON protein sequencer facility, Leiden University.

Denaturation equilibrium transitions

The urea-induced or pH-induced denaturation equilibrium transitions of recombinant βA3- and βA1-crystallin were determined by monitoring the tryptophan fluorescence. The fluorescence was measured on a MPF3L fluorimeter (Perkin Elmer), using an excitation wavelength of 280 nm. For the urea-induced denaturation and renaturation experiments, the fluorescence emission at 355 nm was normalized and used to calculate the fraction of native protein. For the pH-induced denaturation experiments, the change in the wavelength of maximum fluorescence emission (λ_{max}) was used to monitor the denaturation. All samples measured had a final protein concentration of 10 µg/ml and were incubated 24 h at 25°C prior to measurements.

For the urea-denaturation experiments, the proteins were first diluted in 100 mM sodium phosphate, pH 7.0 (SP) to a final concentration of 0.5 mg/ml, and incubated for 2 h at 25°C. Aliquots of 20 µl were then diluted (1/50) in SP buffer containing urea in concentrations ranging from 0 to 8 M. For the urea-renaturation experiments, the proteins were diluted in 100 mM sodium phosphate, pH 7.0, 8 M urea (SPU) to a final concentration of 0.5 mg/ml and incubated for 2 h at 25°C for complete unfolding. Aliquots of 20 µl were refolded by diluting them (1/50) in SP buffer containing urea in concentrations ranging from 0 to 8 M.

For the pH-induced denaturation experiments 20 µl aliquots of either βA3 or βA1, with a protein concentration of 0.5 mg/ml, were diluted (1/50) in buffers with different pH values. Buffers used were: glycine/HCl (pH 1.28, 2.01, 3.05), citric acid/sodium phosphate (pH 3.00, 4.20, 4.80, 6.00, 7.20), Tris/HCl (pH 7.21, 7.80, 9.10) and glycine/NaOH (pH 9.09, 10.27, 11.31, 12.55).

Analytical gel filtration

Gel filtration was performed on a Superose 12 HR 10/30 column (Pharmacia). The column was loaded with protein solutions, containing 100 µg protein (either βA3 or βA1) in 250 µl TEN_{100}, and eluted with TEN_{100} at a flow rate of 0.5 ml/min. Fractions of 500 µl were collected and analysed by SDS-PAGE. Proteins used to correlate molecular weight with elution volume of the Superose 12 column were bovine serum albumin (67 kDa), βB2 (dimer of 46 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (14 kDa).

Analytical ultracentrifugation

Sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Spinco model E analytical ultracentrifuge, equipped with a high intensity light source and a UV scanning system. Experiments were performed with initial protein concentrations of ~150 µg/ml. Double sector cells (12 mm path) with sapphire windows were used in an An F-Ti rotor. High speed sedimentation equilibria were monitored at 280 nm, 12,000 to 16,000 rpm and 24°C. Evaluation from ln c versus r^2 plots made use of a computer program provided by Dr. Gerald Böhm (University of Regensburg). The partial specific volume was calculated from the amino acid composition.

Gel filtration of the water soluble fraction of bovine lens

The water soluble fraction of bovine lens (WSF) was separated on a Superose 6 HR 10/30 gel filtration column (Pharmacia). For this purpose 50 µl of WSF from total lenses of 1.5 year old cows was diluted in 1000 µl of 50 mM Tris-HCl, pH 6.7, 1 mM EDTA, 200 mM KCl (TEK), to a final protein concentration of 6.35 mg/ml. After loading of 1 ml on the Superose 6 column, elution was performed with TEK buffer, at a flow rate of 0.5 ml/min. 1 ml fractions were collected and analysed by SDS-PAGE and by immunoblotting.

^{1}H-NMR spectroscopy of βA3-crystallin

Approximately 50 mg of βA3-crystallin was dissolved in 0.8 ml of 90% H_2O / 10% D_2O containing 25 mM phosphate buffer, pH 7.2 and the pH was adjusted to 5.6 with dilute DCl. ^{1}H-NMR spectra were recorded at 600 MHz and 25°C on a Bruker DMX-600 spectrometer. Spectra were referenced to the residual water resonance at 4.76 ppm. All two-dimensional spectra were acquired in the pure-phase mode using time-proportional phase incrementation (Marion and Wüthrich, 1983) with 512 t1 increments and a sweep width of 6010 Hz over 2048 data points. Through-bond scalar coupling connectivities were determined using total correlation spectroscopy (TOCSY) spectra which were acquired with an MLEV-17 spin lock pulse train with incorporation of delays to minimize effects arising from cross-relaxation (Griesinger *et al.*, 1988). Two TOCSY spectra were acquired with durations for the spin lock mixing periods of 33.8 and 69.2 ms. A NOESY spectrum with a mixing time of 150 ms was used to determine through-space dipolar connectivities (Jeener *et al.*, 1979). In all 2D experiments, water suppression was achieved by a combination of gradient-tailored excitation and pulsed-field gradients using the WATERGATE pulse scheme (Piotto *et al.*, 1992) for the

read pulse. The 2D spectra were processed using Felix software (Biosym). Prior to processing, the data were zero filled to achieve a 2048 x 2048 real matrix and were multiplied by shifted sine-squared bell window functions of 55° to 70° in both dimensions. A ninth-order polynomial baseline correction was applied to the data after processing the t2 domain.

2.4 Results

Expression and characterization of recombinant crystallins

To obtain recombinant βA3 and βA1, the constructs pETβA3 and pETβA1 were transfected into *E. coli* BL21(DE3) as described in Materials and Methods. Samples were taken before and after induction with IPTG, together with samples of the recombinant proteins after purification by anion-exchange chromatography, and analysed by SDS-PAGE (Figure 1A) and immunoblotting (Figure 1B). Despite the use of similar vectors, the expression level of βA1 was much lower (6%) than that of βA3 (15%). The isolated recombinant βA3- and βA1-crystallins were estimated to be 88 and 96% pure, respectively, based on densitometric scanning of Coomassie blue-stained polyacrylamide gels.

N-terminal sequence analyses showed the expected residues for the purified recombinant βA3- and βA1-crystallins, without any evidence of minor truncated forms

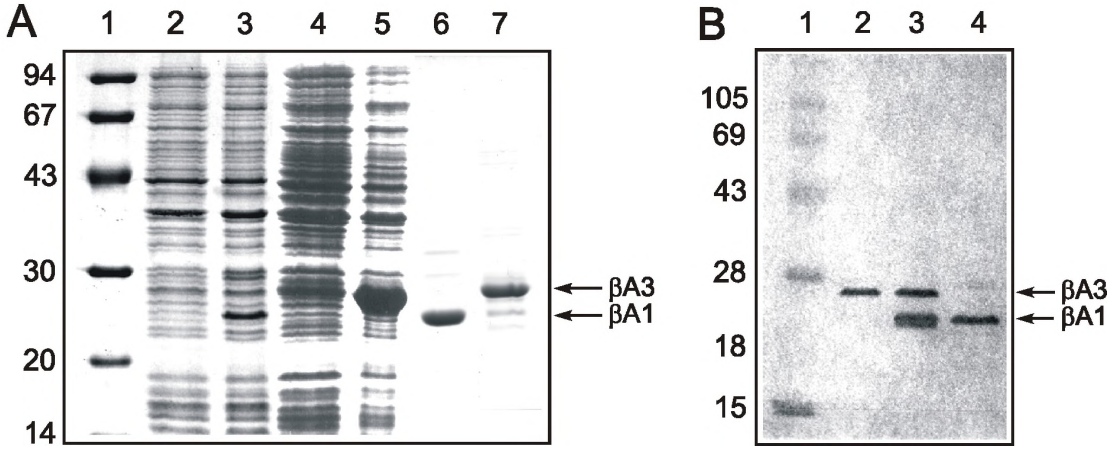


Figure 1. SDS-PAGE showing expression of recombinant βA3- and βA1-crystallin, and the purified proteins after chromatography. (A) Coomassie blue-stained gel: lane 1, low molecular weight markers (LKB); lane 2, the water soluble lysate of non-induced bacteria containing pETβA1; lane 3, same as lane 2, but induced with IPTG; lane 4, the water soluble lysate of non-induced bacteria containing pETβA3; lane 5, same as lane 4, but induced with IPTG; lane 6, purified recombinant βA1; lane 7, purified recombinant βA3. Numbers beside the marker proteins indicate their molecular mass in kDa. (B) Western blot stained with peroxidase using antibodies directed against βA3: lane 1, pre-stained markers; lane 2, water soluble lysate of bacteria expressing βA3; lane 3, water soluble fraction of total bovine lens; lane 4, water soluble lysate of bacteria expressing βA1.

```
                          N-terminal extension          | globular domain
                    1         10         20         30  |
                    |         |          |          |   |
βA3 (predicted)     METQTVQQELESLPTTKMAQTNPMPGSVGP|WKITIY
βA3 (determined)    METQTVQQEL....................|......
βA1 (predicted)                      MAQTNPMPGSVGP|WKITIY
βA1 (determined)                      AQTNPMPGSV..|......
```

Figure 2. N-terminal sequence analysis of recombinant βA3- and βA1-crystallin. Ten steps of Edman degradation were performed.

(Figure 2). Recombinant βA3 retains the initial Met1, as is apparently also the case in the native lens protein (Berbers *et al.*, 1984). The first amino acid of recombinant βA1-crystallin is Ala19 and not Met18, as it is in native βA1. This is not unexpected, because cleavage of the initiation methionine is favoured in prokaryotes when this methionine is followed by a small amino acid (Gly, Ala, Ser, Cys and Thr). These findings confirm the absence of proteolysis during expression in this bacterial system and subsequent purification. Recombinant βA3 expressed in the baculovirus system was found to lack the N-terminal 7 residues and displayed additional proteolytic fragments (Hope *et al.*, 1994). Although our recombinant proteins can be considered full-length, it may be noted, that they differ from the native βA3 and βA1 by the absence of an αN-acetyl group (Berbers *et al.*, 1984).

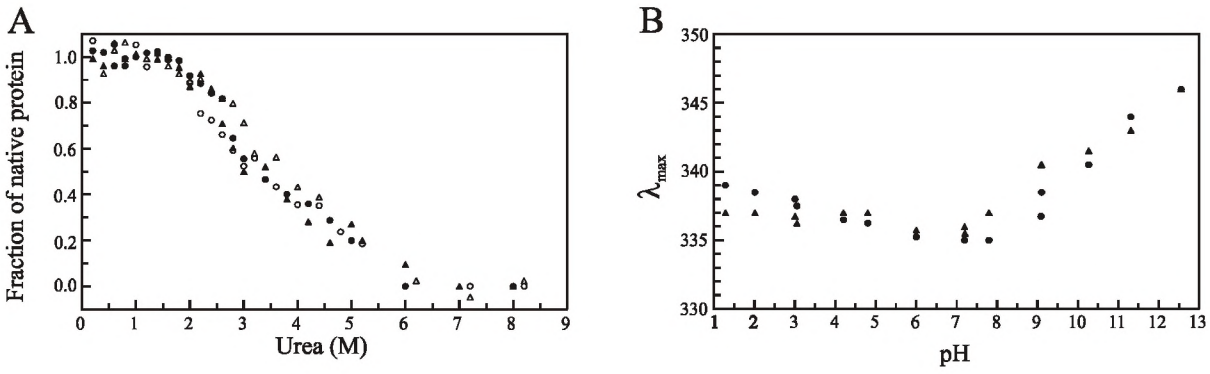


Figure 3. Equilibrium denaturation transitions of recombinant βA3- and βA1-crystallin. (A) Urea-induced equilibrium denaturation transitions. Circles represent βA1 data points; triangles represent βA3 data points. Solid symbols represent data from the denaturation experiments; open symbols represent data from the renaturation experiments. The fraction of native protein was calculated from the normalized fluorescence emission at 355 nm. (B) pH-induced equilibrium denaturation transitions. Circles represent βA1 data points; triangles represent βA3 data points. Both urea-induced and pH-induced equilibrium denaturation transitions show that there is no significant difference in stability between βA3 and βA1.

Denaturation equilibrium transitions

To assess whether the difference in N-terminal extensions has any influence on the stability of the proteins, the denaturation equilibrium transitions of βA3 and βA1 were compared by means of tryptophan fluorescence. Both proteins show similar urea-induced denaturation curves, thus indicating that there is no difference in their stability at pH 7.0 (Figure 3A). The urea-induced denaturation of βA3 and βA1 resembles that of βB2 (Trinkl *et al.*, 1994) in that these β-crystallins are much less stable than γB, which cannot be denatured by urea at this pH (Sharma *et al.*, 1990). The pH-induced denaturation of βA3 and βA1 seems to reveal some minor differences between the two proteins (Figure 3B), however, these differences cannot be considered significant.

Gel filtration and analytical ultracentrifugation

On the Superose 12 column, recombinant βA3 and βA1 migrate as symmetrical peaks at 13.75 and 14.05 ml, respectively, corresponding to molecular masses of 48 and 45 kDa (Figure 4). This is consistent with the expected molecular masses of dimers of βA3 (50 kDa) and dimers of βA1 (46 kDa). The presence of a minor peak just ahead of the dimer peak may indicate a tendency of both proteins to form tetramers. The gel filtration results are confirmed by analytical ultracentrifugation experiments (Figure 5), which yield molecular masses of

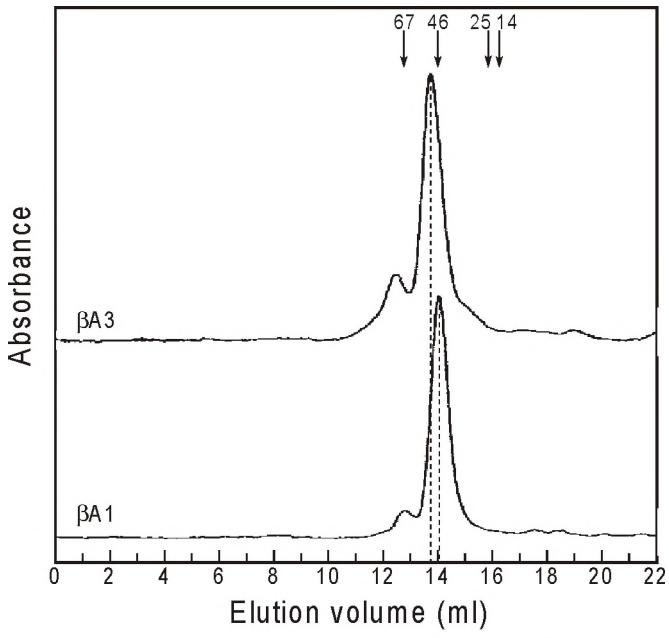


Figure 4. Superose 12 gel filtration chromatography of recombinant βA3- and βA1-crystallin. Arrows indicate the elution volumes of reference proteins. The numbers above the arrows indicate their corresponding molecular mass in kDa. The elution volumes of both βA3 and βA1 correspond to the respective homodimers.

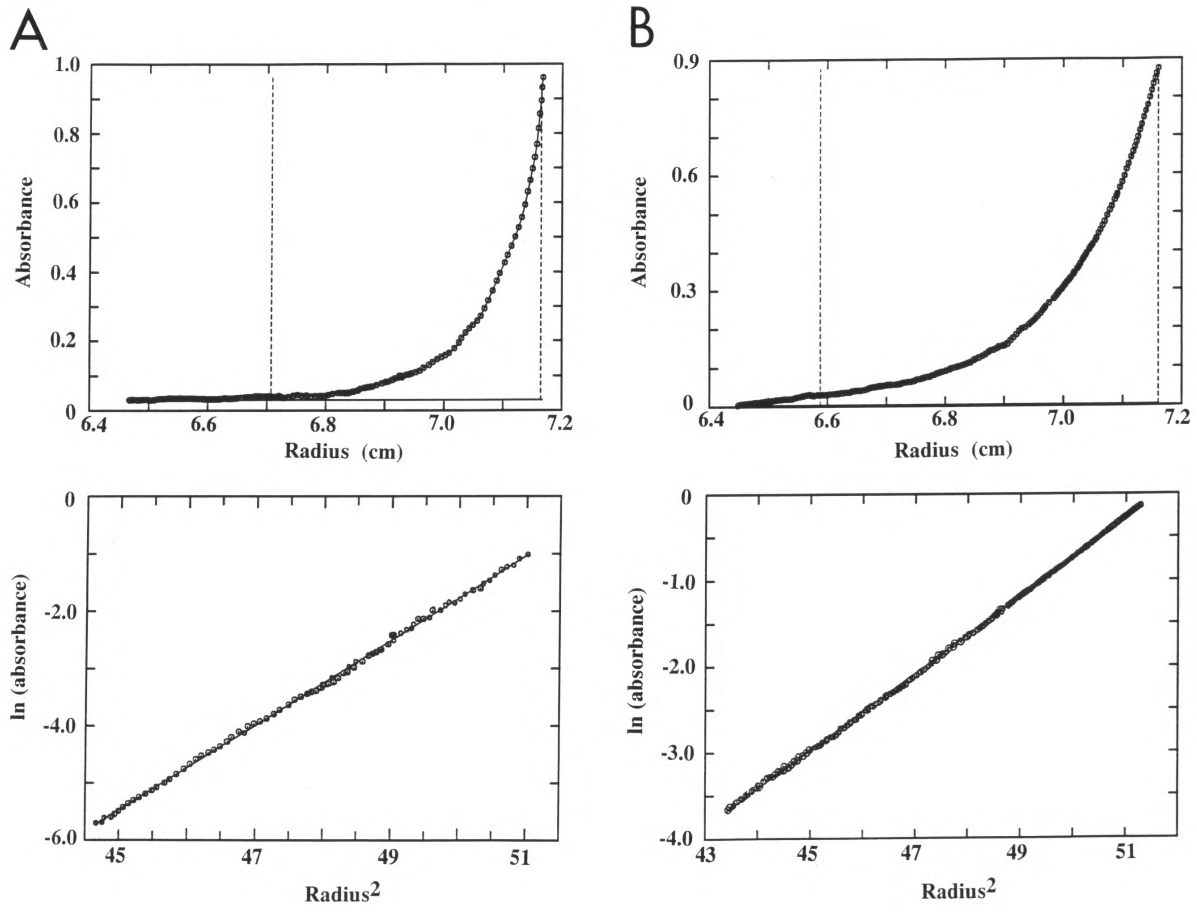


Figure 5. Determination of the molecular masses of (A) βA3 and (B) βA1 by analytical ultracentrifugation. Initial protein concentrations were 150 µg/ml in TEN_{100}. The original data (top) were linearized by plotting the natural logarithm of the absorbance versus the square of the distance. From the slope of the linearized data, the molecular masses of βA3 and βA1 were calculated. The obtained molecular masses of 51.4 ± 2.0 kDa for βA3 and 47.4 ± 1.8 kDa for βA1 correspond to the masses of the respective homodimers.

51.4 ± 2.0 kDa for βA3 and 47.4 ± 1.8 kDa for βA1, again corresponding to dimers. Under these conditions, no indication of tetramer formation was found. The sedimentation coefficients for βA3 and βA1 at zero protein concentration are 4.04 ± 0.08 and 3.94 ± 0.08 S, respectively.

The ability of native βA3 and βA1 to form higher oligomers in the presence of other crystallins was studied in the water soluble fraction of the bovine lens. In the bovine lens, β-crystallin subunits associate into poorly characterized oligomers, ranging from dimers to octamers. Separation of the water soluble fraction of the bovine lens on a Superose 6 column resulted in the appearance of the characteristic molecular weight fractions α, βH, βL1, βL2 and γ (Figure 6A) (Bindels *et al.*, 1981). The Coomassie blue-stained gels show the composition of these fractions (Figure 6B). From the western blots of the Superose 6 fractions it is clear that there is no difference between βA3 and βA1 in their distribution over the

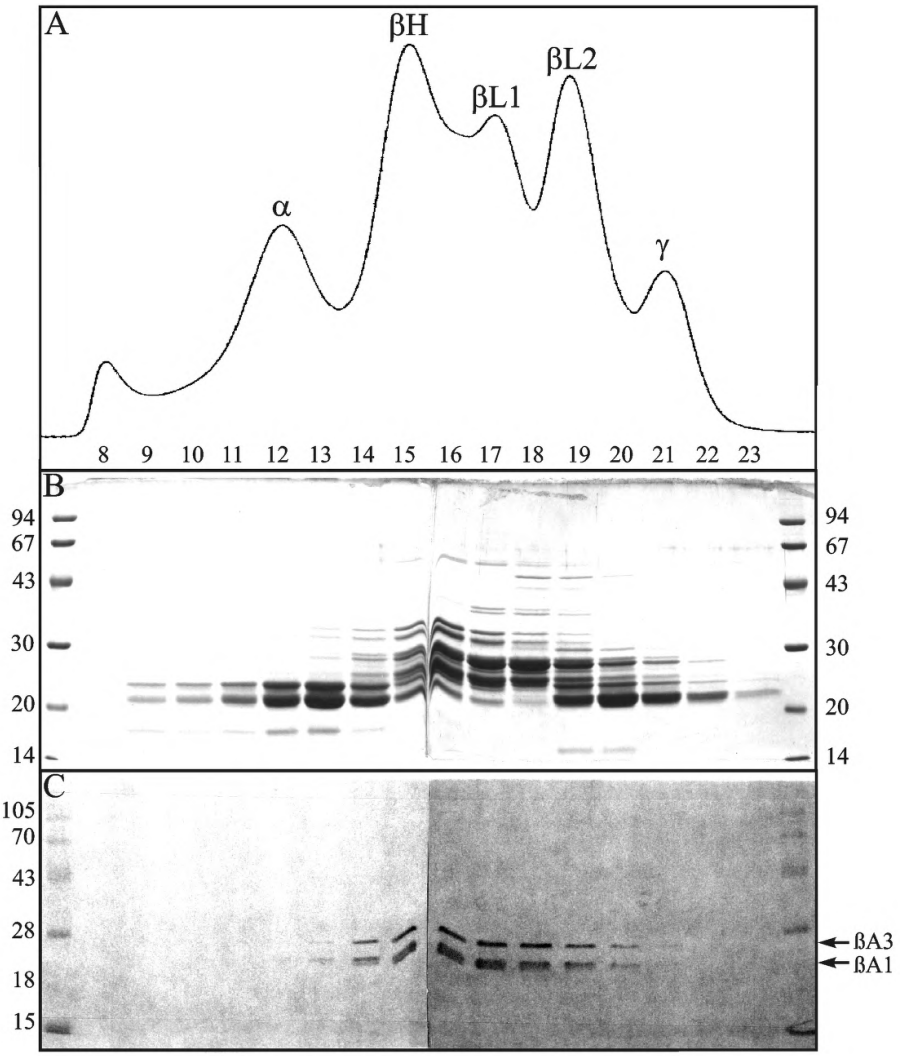


Figure 6. Gel filtration chromatography of water soluble proteins from bovine lens and analysis of the various β-crystallin size classes for the presence of βA3 and βA1. (A) Superose 6 elution profile of the water soluble fraction of total bovine lens. Numbers below the elution profile indicate the analyzed fractions. (B) Coomassie blue-stained polyacrylamide gels of these fractions. Numbers beside the marker proteins indicate their molecular mass in kDa. (C) Corresponding Western blots, stained with peroxidase using an antiserum directed against βA3. The Western blots demonstrate that βA3 and βA1 are present in similar proportions in the fractions βH, βL1 and βL2.

βL1 and βL2, which means that the complexes in which they occur range from dimers to octamers. Most of βA3 and βA1 occurs in the βH and βL1 fraction, indicating their tendency to form higher oligomers rather than dimers. In fact, in earlier studies βA3 was not observed in the dimeric βL2 fraction (Slingsby and Bateman, 1990; Cooper *et al.*, 1993). The Sephacryl S300HR gel filtration column used in those studies may have better separation characteristics than our Superose 6 column. Alternatively, the sensitivity of our Western blotting analysis might well account for this discrepancy. In any case, our results indicate that the difference in length of the N-terminal extensions of βA3 and βA1 does not appear to influence their interaction with other β-crystallins during oligomerization.

^{1}H-NMR spectroscopy of βA3-crystallin

From the presence of sharp resonances in the 1D ^{1}H-NMR spectrum of recombinant βA3 (not shown) it was apparent that one or more regions of relative conformational flexibility were present in the molecule. It was anticipated that these relatively sharp resonances may arise from a flexible N-terminal extension, as was observed in βB2-crystallin (Carver *et al.*, 1993). Indeed, this was found to be the case for βA3 from assignment of the ^{1}H-NMR spectrum, using standard procedures (Wüthrich, 1986), via careful analysis of the 2D TOCSY and NOESY spectra. A series of strong, sequential NOEs between the NH proton and the preceding α-CH resonance were observed from Leu13 to Met1 and from Asn22 to Pro14. No non-sequential NOEs were present, although some sequential NOEs between NH protons and the preceding β-CH protons were observed which assisted in the assignments. As the N-terminus of the recombinant βA3 is not acetylated, the NH resonance of Met1 was not observed owing to exchange with water.

The NH to α-CH, β-CH etc. region of the TOCSY spectrum with a long mixing time shows the assigned cross-peaks for βA3-crystallin (Figure 7). The chemical shift values are given in Table I. It is apparent from Figure 7 that stronger cross-peaks were observed for the region from Glu2 to Leu13 compared to the latter portion of the extension (Thr15 to Asn22), implying that the first portion of the extension has greater flexibility than the region closer to the core of the protein. It was not possible to assign the last seven amino acids in the N-terminal extension (Met24 to Pro30), presumably because they have even less flexibility than the remainder of the extension. Some unassigned, relatively strong cross-peaks were observed in the NH to α-CH region of the 2D spectra (Figure 7), which may arise from some resonances in this portion of the N-terminal extension or other flexible regions in the molecule.

The chemical shift value of the α-CH resonance of an amino acid is a good indicator of what type of secondary structure in which the amino acid is involved in (Wüthrich, 1986; Wishart *et al.*, 1995). Thus, when the α-CH chemical shift values for βA3 were compared with the recent data of Wishart *et al.* (1995) for random coil peptides (including the correction for those amino acids which were followed by prolines, i.e. Leu13 and Asn22), all α-CH resonances in the N-terminal extension of βA3 had chemical shifts very similar to those for a random coil peptide. It is evident, therefore, that like its counterpart in βB2-crystallin, the N-terminal extension of βA3 has little or no ordered structure and it has flexibility that is essentially independent of the domain core of the βA3 dimer. However, in βB2-crystallin only

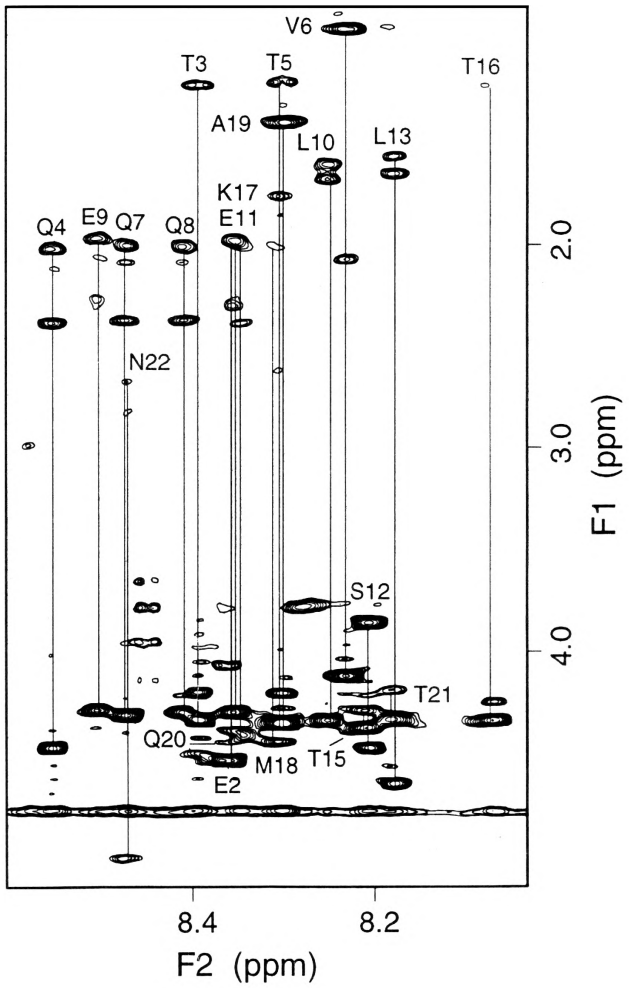


Figure 7. NH to α-CH, β-CH region of a TOCSY spectrum (mixing time = 69.2 ms) of recombinant βA3-crystallin. Assignments determined via the sequential assignment procedure (Wüthrich, 1986) are indicated.

the last two residues of the N-terminal extension, i.e. those that are closest to the domain region, have constrained flexibility as judged by their weak or absent cross-peaks in the NMR spectra (Carver *et al.*, 1993). By contrast, in the N-terminal extension of βA3 the seven residues closest to the globular domain are restricted in mobility.

2.5 Discussion

The sequence extensions of the β-crystallins have been suggested to play a role in their ability to form dimers and higher oligomers, in contrast to the γ-crystallins that are monomeric and lack these extensions (Berbers *et al.*, 1983). Several experimental studies have already been undertaken to investigate the role of these sequence extensions in oligomerization. Trinkl *et al.* (1994) and Kroone *et al.* (1994) have shown that truncation of the N- and/or C-terminal arms of βB2 had no effects on its ability to form homodimers. In contrast, Hope *et al.* (1994)

reported that truncation of the N-terminal 30 residues of βA3 leads to a monomeric protein. However, upon denaturation and renaturation with other lens proteins, this truncated βA3 was able to form heterodimers, but no higher oligomers.

Our findings now show that the absence of 18 amino acids from the N-terminal extension of our recombinant βA1 as compared with βA3, does not affect the overall properties of this protein. βA3 and βA1 are equally stable, as shown by urea- and pH-induced equilibrium denaturation transition measurements. Both βA3 and βA1 form homodimers, and similarly occur in higher oligomers in the presence of other lens crystallins. ^{1}H-NMR spectroscopy of βA3 demonstrates that the larger part of its N-terminal extension, up to and including residue Asn22, has great flexibility and has little or no defined structure. This means that this part of the N-terminal extension, at least in the homodimer, cannot be involved in protein-protein interactions and is able to move freely in the solvent. The observation by Hope *et al.* (1994) that truncation of the complete N-terminal extension of

Table I. Chemical shifts of βA3-crystallin in 90% H_2O / 10% D_2O, pH 5.6, 25°C.

Residue	NH	α-CH	β-CH	Others
Met1	-	4.17	2.19	γ-CH2 = 2.61
Glu2	8.36	4.54	1.98, 2.05	γ-CH2 = 2.29
Thr3	8.40	4.33	4.20	γ-CH3 = 1.22
Gln4	8.56	4.47	2.03, 2.12	γ-CH2 = 2.39
Thr5	8.30	4.35	4.20	γ-CH3 = 1.20
Val6	8.23	4.12	2.07	γ-CH3 = 0.94, 0.94
Gln7	8.47	4.32	2.00, 2.08	γ-CH2 = 2.37
Gln8	8.41	4.30	2.01, 2.09	γ-CH2 = 2.37
Glu9	8.50	4.29	1.97, 2.07	γ-CH2 = 2.27
Leu10	8.25	4.34	1.67	γ-CH2 = 1.60; δ-CH3 = 0.93, 0.93
Glu11	8.36	4.30	1.99, 2.05	γ-CH2 = 2.29
Ser12	8.21	4.47	3.85	-
Leu13	8.18	4.65	1.65	γ-CH2 = 1.57; δ-CH3 = 0.93, 0.93
Pro14	-	4.49	1.98, 2.32	γ-CH2 = 2.08; δ-CH2 = 3.32, 3.59
Thr15	8.21	4.37	4.29	γ-CH3 = 1.24
Thr16	8.07	4.34	4.25	γ-CH3 = 1.21
Lys17	8.31	4.35	1.77, 1.85	γ-CH2 = 1.45; δ-CH2 = 1.70; ε-CH2 = 3.01
Met18	8.31	4.44	2.00	γ-CH2 = 2.54, 2.62
Ala19	8.30	4.35	1.40	-
Gln20	8.35	4.40	2.00, 2.03	γ-CH2 = 2.39
Thr21	8.18	4.33	4.19	γ-CH3 = 1.20
Asn22	8.47	5.01	2.67, 2.82	-

βA3 abolishes dimerization then would indicate that residues 23 to 30 play a crucial role in this interaction. This would mean that the dimer formation and higher-order association of βA3 and βA1 differ fundamentally from the corresponding interactions in βB2. Such an intriguing difference deserves further investigation. It should also be noted in this respect that the N-terminal extension of βB2 retains its flexibility in the various β-crystallin complexes from the bovine lens (βH, βL1, βL2), whereas resonances from other β subunits, including βA3 and βA1, are in general absent from the NMR spectra (Cooper *et al.*, 1993). This indicates that the N-terminal extension of βA3 and βA1, in contrast to that of βB2, is involved in, or constrained by, the association with other β-crystallins. The fact that truncated forms of βA3 and/or βA1, missing 11 and 21 amino acids of the βA3 N-terminal sequence, have been found in cataractous rat lenses (David *et al.*, 1993) indicates, however, that these extensions are accessible to proteolytic enzymes and must thus be solvent exposed.

Our present findings provide no evidence that the mechanism of dual translation initiation, leading to the expression of βA3- and βA1-crystallin, has been conserved in evolution to produce two proteins with different association properties. To find a possible alternative reason for this phenomenon, we compared the theoretical pI values and charge of the N-terminal extensions of βA3 and βA1 in different species. It is shown in Table II that the N-terminal extension of βA3 is always more acidic and more negatively charged than its βA1 counterpart. This conservation is the more remarkable when it is considered that, between species, many mutations have altered the sequence of these extensions (Table II). The charge on proteins is involved in regulating repulsive and attractive interactions between proteins. In the eye lens, where proteins are present in high concentrations, such interactions are especially important (Vérétout *et al.*, 1989; Tardieu *et al.*, 1992). It has been shown that, in

Table II. Comparison of the theoretical charge and pI of the N-terminal extensions of βA3- and βA1-crystallin of different species (Inana *et al.*, 1982; Gorin and Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Hogg *et al.*, 1986; Peterson and Piatigorsky, 1986). The N-terminal extensions of βA3 and βA1 correspond to positions 1 to 30 and 18 to 30, respectively, of the presented protein sequences. Conserved residues across all species are indicated by an asterisk. Charge and pI were calculated using the program PEPSTATS from the GCG-package (Devereux *et al.*, 1984).

Species	N-terminal extension	βA3 (1-30)		βA1 (18-30)	
		Charge	pI	Charge	pI
	1 18 30				
Bovine	METQTVQQELESLPTTKMAQTNPMPGSVGP	-2	4.09	0	6.06
Murine	METQTVQRELETLPTTKMAQTNPMPGSLGP	-1	4.61	0	6.06
Human	METQAEQQELETLPTTKMAQTNPTPGSLGP	-3	3.93	0	6.06
Chicken	MGEAAVPPELDTFPAAKMAQTNPLPVPMGP	-2	4.00	0	6.06
	* ** * ******* * **				

general, changes in charge have been avoided during the evolution of the crystallins (Leunissen *et al.*, 1990). We propose, therefore, that the role of the difference in sequence extensions between βA3- and βA1-crystallins is not in directing oligomerization, or in conferring stability, but in providing these crystallins with a specific charge and pI, which are geared at modulating the short-range non-specific protein interactions in the lens. The negative charge of the βA3 extension would favour repulsive interactions, therefore contributing to lens transparency, whereas the uncharged βA1 extension would allow higher protein concentrations, thereby contributing to the refractive index of the lens. Maintaining these functionally diverse potentials as the product of a single βA3/A1 gene may indeed be selectively advantageous. It would, moreover, make good sense from an evolutionary point of view to use extensions for maintaining differences in charge and pI between βA3 and βA1, because the extensions are solvent-exposed and directly change the charge of the molecule as a whole.

2.6 Acknowledgements

We wish to thank Dr. Graham Ball for assistance with acquisition of the NMR spectra, and Dr. Eva-Maria Mayr and Stefan Palme for their help with the denaturation experiments. This work was supported by EC-HCM grant CHRX-CT93-0175 and by a grant to J.C. from the National Health and Medical Research Council of Australia.

2.7 References

Bax, B., Lapatto, R., Nalini, V., Driessen, H., Lindley, P.F., Mahadevan, D., Blundell, T.L. and Slingsby, C. (1990) *Nature*, **347**, 776-780.

Benedek, G.B. (1971) *Appl. Optics*, **10**, 459-473.

Berbers, G.A.M., Boerman, O.C., Bloemendal, H. and de Jong, W.W. (1982) *Eur. J. Biochem.*, **128**, 495-502.

Berbers, G.A.M, Brans, A.M., Hoekman, W.A., Slingsby, C., Bloemendal, H. and de Jong, W.W. (1983) *Biochim. Biophys. Acta*, **748**, 213-219.

Berbers, G.A.M., Hoekman, W.A., Bloemendal, H., de Jong, W.W., Kleinschmidt, T. and Braunitzer, G. (1984) *Eur. J. Biochem.* **139**, 467-479.

Bindels, J.G., Koppers, A. and Hoenders, H.J. (1981) *Exp. Eye Res.* **33**, 333-343.

Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B. and Wistow, G. (1981) *Nature*, **289**, 771-777.

Carver, J.A., Cooper, P.G. and Truscott, R.J.W. (1993) *Eur. J. Biochem.*, **213**, 313-320.

Cooper, P.G., Carver, J.A. and Truscott, R.J.W. (1993) *Eur. J. Biochem.*, **213**, 321-328.

Cooper, P.G., Carver, J.A., Aquilina, J.A., Ralston, G.B. and Truscott, R.J.W. (1994) *Exp. Eye Res.*, **59**, 211-220.

David, L.L., Shearer, T.R. and Shih, M. (1993) *J. Biol. Chem.*, **268**, 1937-1940.

Delaye, M. and Tardieu, A. (1983) *Nature*, **302**, 415-417.

Devereux, J., Haeberli, P. and Smithies, O. (1984) *Nucl. Acids Res.*, **12**, 387-395.

Driessen, H.P.C., Herbrink, P., Bloemendal, H. and de Jong, W.W. (1981) *Eur. J. Biochem.*, **121**, 83-91.

Gorin, M.B. and Horwitz, J. (1984) *Curr. Eye Res.*, **3**, 939-948.

Griesinger, C., Otting, G., Wüthrich, K. and Ernst, R.R. (1988) *J. Am. Chem. Soc.*, **110**, 7870-7872.

Groenen, P.J.T.A., Grootjans, J.J., Lubsen, N.H., Bloemendal, H. and de Jong, W.W. (1994) *J. Biol. Chem.*, **269**, 831-833.

Hogg, D., Tsui, L.C., Gorin, M. and Breitmann, M.L. (1986) *J. Biol. Chem.*, **261**, 12410-12427.

Hope, J.N., Hao-Chia, C. and Hejtmancik, J.F. (1994) *Protein Engng.*, **7**, 445-451.

Inana, G., Shinohara, T., Maizel, J.V. and Piatigorsky, J. (1982) *J. Biol. Chem.*, **257**, 9064-9071.

Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R.R. (1979) *J. Chem. Phys.*,**71**, 4546-4553.

Kozak, M. (1989) *J. Cell. Biol.*, **108**, 229-241.

Kozak, M. (1991) *J. Cell. Biol.*, **115**, 887-903.

Kroone, R.C., Elliott, G.S., Ferszt, A., Slingsby, C., Lubsen, N.H. and Schoenmakers, J.G.G. (1994) *Protein Engng.*, **7**, 1395-1399.

Lapatto, R., Nalini, V., Bax, B., Driessen, H., Lindley, P.F., Blundell, T.L. and Slingsby, C. (1991) *J. Mol. Biol.*, **222**, 1067-1083.

Leunissen, J.A.M., van den Hooven, H.W. and de Jong, W.W. (1990) *J. Molec. Evol.*, **31**, 33-39.

Lubsen, N.H., Aarts, H.J.M. and Schoenmakers, J.G.G. (1988) *Prog. Biophys. Mol. Biol.*, **51**, 47-76.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **113**, 967-974.

Peterson, C.A. and Piatigorsky, J. (1986) *Gene*, **45**, 139-147.

Piotto, M., Saudek, V. and Sklenar, V. (1992) *J. Biomol. NMR*, **2**, 661-665.

Quax-Jeuken, Y., Janssen, C., Quax, W., van den Heuvel, R. and Bloemendal, H. (1984) *J. Mol. Biol.*, **180**, 457-472.

Quax-Jeuken, Y., Driessen, H., Leunissen, J., Quax, W., de Jong, W.W. and Bloemendal, H. (1985) *EMBO J.*, **4**, 2597-2602.

Sharma, A., Minke-Gogl, V., Gohl, P., Siebendritt, R., Jaenicke, R. and Rudolph, R. (1990) *Eur. J. Biochem.*, **194**, 603-609.

Slingsby, C. and Bateman, O.A. (1990) *Exp. Eye Res.*, **51** 21-26.

Slingsby, C., Driessen, H.P.C., Mahadevan, D., Bax, B. and Blundell, T.L. (1988) *Exp. Eye Res.*, **46**, 375-403.

Tardieu, A., Vérétout, F., Krop, B. and Slingsby, C. (1992) *Eur. Biophys. J.*, **21**, 1-12.

Trinkl, S., Glockshuber, R. and Jaenicke, R. (1994) *Protein Sci.*, **3**, 1392-1400.

Vérétout, F. and Tardieu, A. (1989) *Eur. Biophys. J.*, **17**, 61-68.

White, H.E., Driessen, H.P.C., Slingsby, C., Moss, D.S., Lindley, P.F. (1989) *J. Mol. Biol.*, **207**, 217-235.

Wishart, D.S., Bigam, C.G., Holm, A., Hodges, R.S. and Sykes, B.D. (1995) *J. Biomol. NMR*, **5**, 67-81.

Wistow, G.J. and Piatigorsky, J. (1988) *Annu. Rev. Biochem.*, **57**, 479-504.

Wistow, G., Slingsby, C., Blundell, T., Driessen, H., de Jong, W. and Bloemendal, H. (1981) *FEBS Lett.*, **133**, 9-16.

Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P. and Blundell, T. (1983) *J. Mol. Biol.*, **170**, 175-202.

Wüthrich, K. (1986) *NMR of proteins and nucleic acids*. J. Wiley, New York.

CHAPTER III

Truncation of βA3/A1-crystallin during aging of the bovine lens; possible implications for lens optical quality

Paul J.L. Werten, Erwin Vos & Wilfried W. de Jong

Experimental Eye Research **68**, 99-103.

3.1 Summary

During aging, extensive modifications of eye lens proteins take place, which may contribute to the development of cataract. Truncation of the accessible extensions of β-crystallins has been suggested to be an important factor in this process. We therefore studied the truncations of bovine βA3- and βA1-crystallin in more detail. These proteins are identical except for the length of their N-terminal extension, 30 and 13 residues, respectively. The water-soluble and -insoluble proteins from cortex and nucleus of bovine lenses of different ages were separated by 2D gel electrophoresis and immuno-blotted with an antiserum against βA3. Two major truncation products were detected, which by sequence analysis were found to correspond to βA3 having lost 11 or 22 amino acids. βA3(-11) was only observed in the nucleus, whereas βA3(-22) was present both in cortex and nucleus. We argue, therefore, that each of these two products is produced by a separate proteolytic enzyme. βA3(-22) can originate by cleavage of βA3, βA1 and βA3(-11). Truncation of βA3 occurs more readily than that of βA1, while βA3(-11) disappears at an intermediate rate. It appears that the longer the N-terminal extension, the easier proteolysis takes place. Truncated proteins are not necessarily prone to end up in the water-insoluble fractions; other modifications leading to charge changes are more likely to be responsible for insolubilization. Truncation of the extensions of β-crystallins could be a functional rather than a harmful process during aging of the lens; by modulating protein repulsion, it may help to maintain the protein concentration gradient that is necessary for the optical quality of the lens.

3.2 Introduction

Transparency and refractive index of the vertebrate eye lens depend on the short-range interactions and concentrations of the water-soluble proteins in the lens fiber cells, respectively (Benedek, 1971; Delaye and Tardieu, 1983). Post-translational modifications, which accumulate during aging, may disturb the normal interactions between these proteins and may eventually lead to cataract formation. The β-crystallins can be major targets for such modifications. These oligomeric structural lens proteins have a two-domain structure -- each domain consisting of two so-called Greek key β-sheet motifs -- and variable N- and sometimes C-terminal extensions (Blundell *et al.*, 1981; Driessen *et al.*, 1981; Wistow *et al.*, 1983; Bax *et al.*, 1990). The extensions may play a role in stabilizing oligomers of these proteins (Berbers *et al.*, 1983; Hope *et al.*, 1994; Sergeev *et al.,* 1997) or, alternatively, act as

physical spacers preventing further oligomerization (Trinkl *et al.*, 1994). A third possibility is that they act as solvent exposed "carriers of charge" (Werten *et al.*, 1996), regulating the short-range, non-specific protein interactions in the lens.

The extensions of β-crystallins are susceptible to proteolysis during aging, which may be one of the processes leading to opacification of the lens (Pierscionek and Augusteyn, 1988; Harding, 1991; David *et al.*, 1994). In this study we have investigated the proteolysis of two specific β-crystallins, βA3 and βA1, in more detail. These two proteins are produced from the single βA3/A1 mRNA by a process known as dual translation initiation (Kozak, 1989, 1991). Because the two start codons are in the same reading frame, the resulting proteins are identical, except that βA3 has a 17 residue longer N-terminal extension than βA1. Being present in mammals and birds (Inana *et al.*, 1982; Gorin and Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Hogg *et al.*, 1986; Peterson and Piatigorsky, 1986), this rare feature must have been conserved during evolution for at least 250 million years. This indicates that there must be a functional advantage for having these two different extensions attached to otherwise identical proteins. By identifying the age-dependent cleavage products of βA3/A1-crystallin in the bovine lens, and observing the relative susceptibility of βA3 and βA1 to proteolysis, we attempted to reconstruct the proteolytic pathways involved. The observations are interpreted with regard to the possible implications for the optical quality of the lens, as well as for cataract development.

3.3 Methods

2D-gel electrophoresis and Western blotting of bovine lens proteins

Bovine lenses, from 0.4 to 4.0 years of age, were divided into outer cortical and inner nuclear fractions. Each of these fractions was homogenized in a buffer containing 20 mM NaH_2PO_4/Na_2HPO_4 (pH 6.9), 1 mM EDTA, 100 mM Na_2SO_4 and further separated into a water-soluble fraction (WSF) and a water-insoluble fraction (WIF) by centrifugation at 13,000 rpm and 4°C for 45 min (Sorvall SA-600). The water-insoluble fractions were dissolved in the same buffer containing 6M urea, and re-centrifuged to remove all urea-insoluble material. All fractions were analyzed by means of 2D-gel electrophoresis and subsequent Western blotting. Approximately 1 µg of total protein was loaded on each gel. Proteins were separated using equilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE (13%) in the second dimension. Cleavage products of βA3/A1-crystallin

were detected by using a 1:10,000 dilution of a polyclonal antiserum raised against recombinant βA3-crystallin in rabbits. Alkaline phosphatase conjugated antibodies directed against rabbit IgG (Promega) were used for staining.

Identification of cleavage sites in βA3/A1

To identify the cleavage sites of the major cleavage products of βA3/A1, approximately 100 µg of protein from the WSF of 0.4-year-old nucleus was separated by 2D-gel electrophoresis, and subsequently blotted onto PVDF-membrane (Immobilon-P, Millipore). The PVDF membrane was stained with Coomassie Brilliant Blue and partially destained. The spots corresponding to the two major cleavage products were excised from the PVDF-membrane and destained further. N-terminal sequencing of these fragments was performed at the SON protein sequencer facility, Leiden University.

3.4 Results and Discussion

Identification of cleavage products of βA3/A1

To establish the temporal and spatial localization of proteolysis of βA3/A1 in the aging bovine lens, 0.4-, 1.4- and 4.0-year-old lenses were first divided into cortical and nuclear fractions, and subsequently into water-soluble and urea-soluble fractions. These fractions, twelve in total, were then subjected to 2D-gel electrophoresis, blotted onto nitrocellulose (Hybond-C pure, Ammersham) and immuno-stained, using an antiserum raised against βA3. This antiserum specifically recognizes βA3, βA1 and their post-translational modification products. Figure 1 gives an overview of the obtained patterns. To interpret these patterns, we first identified the two major cleavage products of βA3/A1, which are already conspicuously present in the water-soluble fraction of 0.4-year-old nucleus (see arrowheads in Fig. 1, panel 0.4 y / WSF nucleus). These two proteins were isolated from this fraction by 2D-gel electrophoresis and blotting onto PVDF membrane. After excision and automated Edman degradation, their N-terminal amino acid sequences were found to correspond to βA3 missing 11 or 22 amino acids from its N-terminal extension (Fig. 2). The βA3(-22) product could also originate from βA1 by cleaving five amino acids off its N-terminal extension. The sequencing strategy described above could not be used to identify the other and often minor degradation products of βA3/A1. Since upon aging the modification pattern rapidly becomes more complicated, especially on Coomassie Brilliant Blue-stained PVDF membrane, these products

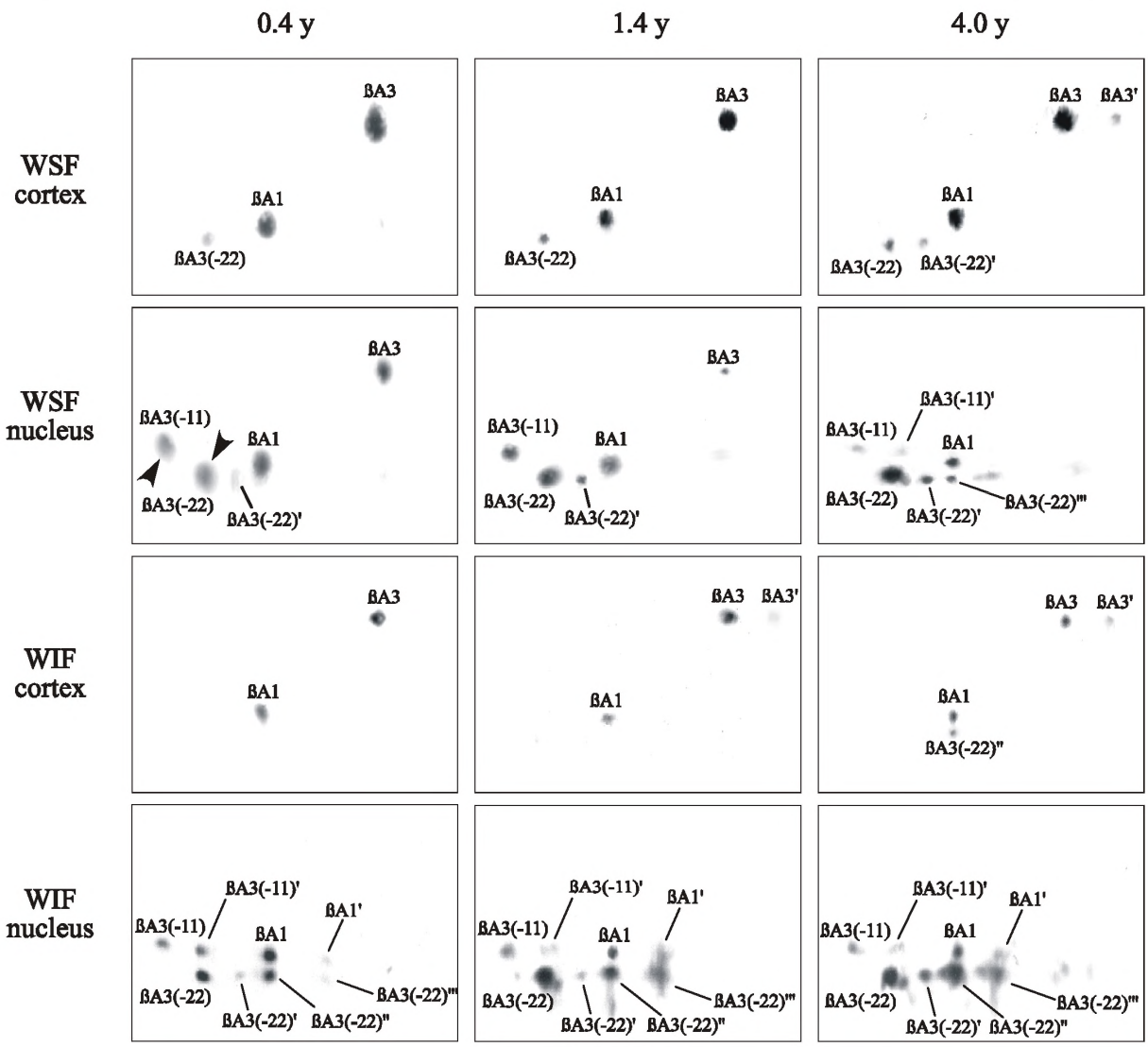


Figure 1. Western blots of 2D-gels of bovine lens proteins. Bovine lenses of 0.4-, 1.4- and 4.0-year-old cows were divided into cortical and nuclear fractions, and subsequently into water-soluble (WSF) and -insoluble fractions (WIF). After 2D-gel electrophoresis and Western blotting of these fractions, βA3, βA1 and their post-translational modification products were immuno-stained with an antiserum that specifically recognizes these proteins. This figure only shows that region of the Western blots where the β-crystallins migrate, with the acidic side to the right and the basic side to the left. For modification products of βA3/A1, see text.

could not be obtained sufficiently pure to warrant reliable Edman degradation. Based upon size and charge, relative to the four known forms (βA3, βA1, βA3(-11) and βA3(-22)), assumptions about their identity were made (see below).

The natural occurrence of both βA3(-11) and βA3(-22) in bovine lenses was recently independently demonstrated by Shih *et al.* (1998). However, that study did not describe the precise temporal and spatial distribution of these truncation products in the bovine lens, which is the main subject of the present work.

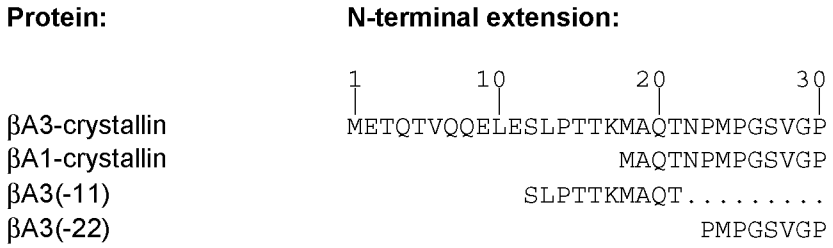


Figure 2. Sequences of the N-terminal extension of bovine βA3- and βA1-crystallin, and identification of the two major βA3/A1 cleavage products, βA3(-11) and βA3(-22), as determined by amino acid sequencing. Ten steps of Edman degradation were performed on the cleavage products.

Age-dependent changes of βA3/A1 in water-soluble bovine lens fractions

During aging, relatively little happens in the cortical WSF with regard to the composition and relative amounts of the proteins present (Fig. 1; see Table I for quantifications). This is to be expected, since eye lenses continue to grow throughout life, and the cortex therefore always contains the most recently synthesized crystallins and the lowest amount of cleavage products. Nevertheless, in the cortical WSF of 0.4-year-old lenses, βA3(-22) can already be detected in small amounts, next to unmodified βA3 and βA1. The amount of βA3(-22) slightly increases in the 1.4- and 4.0-year-old fractions. The relative amounts of βA3 and βA1 remain almost the same (Table I), consistent with the low amount of cleavage products in these fractions. In the 4.0-year-old fraction there are some first indications of modified forms of βA3 and βA3(-22) (indicated as βA3' and βA3(-22)', respectively, in Fig. 1), not arising from proteolysis but most likely from deamidations or other acidifying post-translational modifications.

In the 0.4-year-old nuclear WSF, we see that the amount of βA3 has considerably decreased when compared to the cortical WSF, while the amount of βA1 remained the same. At the same time βA3(-22) has increased and βA3(-11) has appeared. This loss of βA3 becomes even more obvious in the 1.4-year-old fraction. Here, as before in the 0.4-year-old fraction, the amount of βA1 remained virtually the same, whereas βA3 has almost completely disappeared. Coinciding with the disappearance of βA3 are the increasing amounts of βA3(-11) and βA3(-22). The amount of βA3(-22)', which is already vaguely visible in the 0.4-year-old fraction, has also increased. In the 4.0-year-old fraction, βA3 can no longer be detected. The amount of βA3(-11) is strongly decreased too, while the amount of βA3(-22) is still increasing (Table I). A modified form of βA3(-11) (indicated as βA3(-11)' in Fig. 1) and a second modified form of βA3(-22) (indicated as βA3(-22)'' in Fig. 1) have appeared, showing that this older material has undergone much more post-translational modification.

TABLE I. Quantification of βA3, βA1 and their post-translational modification products by densitometric scanning of the Western blots of Figure 1*

Fraction	Protein	0.4 years	1.4 years	4.0 years
WSF cortex	βA3	61 %	61 %	55 %
	βA1	35 %	34 %	31 %
	βA3(-22)	4 %	5 %	7 %
	βA3'	0 %	0 %	4 %
	βA3(-22)'	0 %	0 %	3 %
WSF nucleus	βA3	23 %	4 %	0 %
	βA1	33 %	28 %	18 %
	βA3(-11)	16 %	20 %	4 %
	βA3(-22)	25 %	42 %	52 %
	βA3(-11)'	0 %	0 %	2 %
	βA3(-22)'	3 %	6 %	12 %
	βA3(-22)''	0 %	0 %	7 %
	others	0 %	0 %	5 %

* It should be noted that these quantifications do not represent exact amounts, since intensity of the staining may not be linear with concentration, and can vary for each protein (the antiserum is slightly more reactive to βA3 as compared to βA1) and even between blots (depending on blotting efficiency and the time used for staining). This table is meant to facilitate the interpretation of Figure 1.

Only at this age, where βA3 is completely missing and βA3(-11) has almost disappeared, does the amount of βA1 start to decrease. This strongly indicates that the longer the N-terminal extension of these proteins (βA3 > βA3(-11) > βA1), the more susceptible these proteins are for proteolysis at position 22 of the βA3/A1 sequence. It also shows that, in the end, almost all βA3/A1 becomes βA3(-22) and isoforms thereof. This has been summarized in Figure 3, where a schematic representation shows the putative modification routes involved.

When comparing the proteins present in the cortical and nuclear WSF, it is intriguing that βA3(-11) can only be detected in the nucleus, whereas βA3(-22) is present both in nucleus and cortex. The spatial separation of these two different cleavage products indicates that there might be two different proteolytic enzymes involved in the cleavages at positions 11 and 22. After all, if the same enzyme were responsible for both cleavages, βA3(-11) should be present in the cortical WSF as well. Indeed, the recent results by Shih *et al.* (1998) also suggest that two enzymes are involved. It had been shown previously that m-calpain is most likely responsible for a whole range of cleavage products in rat lenses during cataract formation and normal lens maturation (David *et al.*, 1994). However, Shih *et al.* (1998) now have shown that although m-calpain is able to produce βA3(-11) when added to fetal calf lens proteins *in vitro*, it is unable to produce βA3(-22).

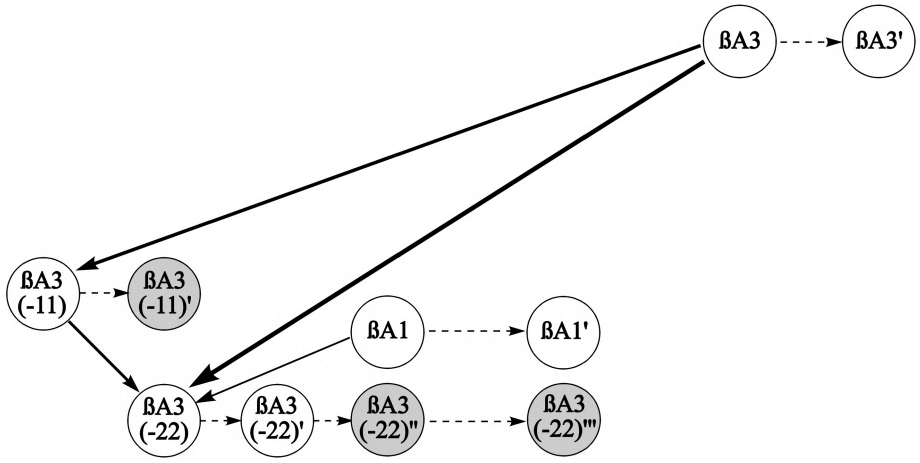


Figure 3. Schematic representation of the major modification routes of βA3 and βA1 during aging of the bovine lens. Proteolysis is indicated by drawn lines. Modifications leading to charge differences are indicated by broken lines. In the case of proteolysis, the thickness of the lines indicates the predominance of the process. Proteins that were found almost exclusively in the water-insoluble fraction are shown in gray circles. Proteolytic routes, which are shown here only between the unmodified proteins, may similarly also exist between the modified proteins.

Age-dependent changes of βA3/A1 in water-insoluble bovine lens fractions

Before we evaluate the changes occurring in the water-insoluble bovine lens fractions, it should first be noted that these fractions only contain a very small part of the total protein content of the respective bovine lenses. It has been shown by Bours *et al.* (1976) that in the cortical fractions of 0.16-year-old bovine lenses only 2% of the total lens protein is insoluble. This increases up to 15% in 18.7-year-old nuclear lens fractions. In the fractions analyzed in our study, isolated from bovine lenses ranging from 0.4 to 4.0 years of age, only a very minor part of the total lens protein will therefore be water-insoluble. The fact that similar amounts of protein were loaded for all fractions on the 2D gels shown in Figure 1 may falsely give the impression that the actual amount of water-insoluble proteins present in bovine lenses is very high. This is most certainly not the case.

It should also be noted that, in theory, the βA3/A1 related proteins present in the water-insoluble fractions could not only have been insolubilized due to changes in their own structure, but also due to changes in other β-crystallin subunits present in the same heteromeric complexes. It is not known whether 'healthy' subunits can 'escape' from complexes that become insoluble, however, it has been shown that β-crystallin subunits can exchange spontaneously to form new complexes (Slingsby *et al.*, 1994; Hejtmancik *et al.*,

1997). We suggest therefore that, overall, the proteins found in the water-insoluble fractions are mainly insolubilized due to changes in their own structure.

Turning then to the cortical WIF, it can be seen that these fractions contain mostly βA3 and βA1, as do the corresponding water-soluble cortical fractions. We failed to detect βA3(-22) in these fractions, which clearly is present in the water-soluble cortical fractions. This indicates that truncation of the β-crystallin arms does not necessarily result in less soluble protein complexes *per se*. Moreover, it is interesting to find that in the 4.0-year-old WIF, βA3(-22)'' starts appearing, whereas βA3(-22) itself or its modified form βA3(-22)' cannot be detected. This might indicate that insolubilization of β-crystallin complexes is caused by charge modifications that occur in the globular domains of the proteins which disrupt protein structure, rather than by truncations. This seems to be confirmed by the fact that in the nuclear water-insoluble fractions proteins like βA3(-22)'' and βA3(-11)' appear to be over-represented as compared to the corresponding water-soluble fractions. Indeed, many further post-translationally modified isoforms (notably βA3(-22)''' and βA1') are heavily accumulating in the water-insoluble fractions.

Implications for lens optical quality and cataract development

Based on our observations it can be argued that truncation of β-crystallin extensions, as occurring in βA3/A1 during lens maturation, is a process that can be of functional value to the lens, rather than causing protein insolubilization and cataract. In the case of bovine βA3, the N-terminal extension is 30 amino acids long and carries negative charges that are lost upon truncation. Given that β-crystallin extensions may play an important role as physical spacers or as carriers of charge that regulate the short-range, non-specific protein interactions in the lens, truncation would cause the loss of spacers and/or repulsive forces in βA3-containing complexes. This could then lead to a closer packing of the β-crystallin complexes in regions where this truncation occurs. Truncation seems to gradually increase from cortex to nucleus, coinciding with the necessity for tighter packing of proteins towards the center of the lens in order to maintain its required refractive properties. We therefore suggest that, at least for βA3, the truncation of its N-terminal extension may very well contribute to establishing a gradually higher protein concentration towards the center of the lens.

3.5 Acknowledgments

We thank Drs. Martinus van Boekel for initial help, Johan Bours for supplying bovine lenses of different ages, and Larry David for useful exchange of information. This work was supported by EC-HCM grant CHRX-CT93-0175 and the Netherlands Foundation for Chemical Research (SON).

3.6 References

Bax, B., Lapatto, R., Nalini ,V., Driessen, H., Lindley, P.F., Mahadevan, D., Blundell, T.L. and Slingsby, C. (1990). X-ray analysis of βB2-crystallin and evolution of oligomeric lens proteins. *Nature* **347**, 776-780.

Benedek, G.B. (1971). Theory of transparency of the eye. *Appl. Opt.* **10**, 459-473.

Berbers, G.A., Brans, A.M., Hoekman, W.A., Slingsby, C., Bloemendal, H. and W.W. de Jong (1983). Aggregation behavior of the bovine β-crystallin Bp chain studied by limited proteolysis. *Biochim. Biophys. Acta* **748**, 213-219.

Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B. and Wistow, G. (1981). The molecular structure and stability of the eye lens: X-ray analysis of γ-crystallin II. *Nature* **289**, 771-777.

Bours, J., Doepfmer, K. and Hockwin, O. (1976). Isoelectric focusing of crystallins from different parts of the bovine and dog lens in dependence of age. *Doc. Ophthalmol. Proc. Ser.* **8**, 75-98.

David, L.L., Azuma, M. and Shearer, T.R. (1994). Cataract and the acceleration of calpain-induced β-crystallin insolubilization occurring during normal maturation of rat lens. *Invest. Ophthalmol. Vis. Sci.* **35**, 785-793.

Delaye, M. and Tardieu, A. (1983). Short-range order of crystallin proteins accounts for eye lens transparency. *Nature*, **302**, 415-417.

Driessen, H.P.C., Herbrink, P., Bloemendal, H. and de Jong, W.W. (1981). Primary structure of the bovine β-crystallin Bp chain. Internal duplication and homology with γ-crystallin. *Eur. J. Biochem.* **121**, 83-91.

Gorin, B. and Horwitz, J. (1984). Cloning and characterization of a cow beta crystallin cDNA. *Curr. Eye Res.* **3**, 939-948.

Harding, J. (1991). Common pathways to cataract. In: *Cataract Biochemistry Epidemiology and Pharmacology.* London: Chapman & Hall; 1991:191.

Hejtmancik, J.F., Wingfield, P.T., Chambers, C., Russell, P., Chen, H.C., Sergeev, Y.V. and Hope, J.N. (1997). Association properties of βB2- and βA3-crystallin: ability to form dimers. *Protein Eng.* **10**, 1347-1352.

Hogg, D., Tsui, L.C., Gorin, M. and Breitman, M.L. (1986). Characterization of the human β-crystallin gene HuβA3/A1 reveals ancestral relationships among the βγ-crystallin superfamily. *J. Biol. Chem.* **261**, 12420-12427.

Hope, J.N., Chen, H.C. and Hejtmancik, J.F. (1994). βA3/A1-crystallin association: Role of the N-terminal arm. *Protein Eng.* **7**, 445-451.

Inana, G., Shinohara, T., Maizel, J.V.J. and Piatigorsky, J. (1982). Evolution and diversity of the crystallins. Nucleotide sequence of a β-crystallin mRNA from the mouse lens. *J Biol. Chem.* **257**, 9064-9071.

Kozak, M. (1989). The scanning model for translation: an update. *J. Cell. Biol.* **108**, 229-241.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell. Biol.* **115**, 887-903.

Peterson, C.A. and Piatigorsky, J. (1986). Preferential conservation of the globular domains of the βA3/A1-crystallin polypeptide of the chicken eye lens. *Gene* **45**, 139-147.

Pierscionek, B. and Augusteyn, R.C. (1988). Protein distribution patterns in concentric layers from single bovine lenses: changes with development and ageing. *Curr. Eye Res.* **7**, 11-23.

Quax-Jeuken, Y., Janssen, C., Quax, W., van den Heuvel, R. and Bloemendal, H. (1984). Bovine β-crystallin complementary DNA clones. Alternating proline/alanine sequence of βB1 subunit originates from a repetitive DNA sequence. *J. Mol. Biol.* **180**, 457-472.

Sergeev, Y., Wingfield, P., Hope, J. and Hejtmancik, J.F. (1997). The role of C-terminal extension in βB2-crystallin oligomerization. *Invest. Ophtalmol. Vis. Sci.* **38**, S206.

Shih, M., Lampi, K.J., Shearer, T.R., and David, L.L. (1998). Cleavage of β-crystallins during maturation of bovine lens. *Mol. Vis.* **4**, 4 <http://www.emory.edu/molvis/v4/p4>.

Slingsby, C. and Bateman, O.A. (1994). Formation and crystallization of the eye lens heterodimer βB2-βB3-crystallin. *Exp. Eye Res.* **58**, 761-764.

Trinkl, S., Glockshuber, R. and Jaenicke, R. (1994). Dimerization of βB2-crystallin: The role of the linker peptide and the N- and C-terminal extensions. *Protein Sci.* **3**, 1392-1400.

Werten, P.J.L., Carver, J.A., Jaenicke, R. and de Jong, W.W. (1996). The elusive role of the N-terminal extension of βA3- and βA1-crystallin. *Protein Eng.* **9**, 1021-1028.

Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P. and Blundell, T. (1983). X-ray analysis of the eye lens protein γII-crystallin at 1.9 Å resolution. *J. Mol. Biol.* **170**, 175-202.

CHAPTER IV

The short 5' untranslated region of the βA3/A1-crystallin mRNA is responsible for leaky ribosomal scanning

Paul J.L. Werten, Gerard J.J. Stege & Wilfried W. de Jong

Molecular Biology Reports **26**, 201-205.

4.1 Abstract

Leaky ribosomal scanning allows the expression of multiple proteins from a single mRNA by occasionally skipping the first start codon, and initiating translation at a subsequent one. βA3- and βA1-crystallin, two members of the β-crystallin family of vertebrate eye lens proteins, are produced via this mechanism, which is only very rarely used in eukaryotic genes. Since the two start codons lie in the same reading frame on the βA3/A1 messenger, the two translated proteins are identical, except for the 17 residues shorter N-terminal extension of βA1-crystallin. It has been suggested that the very short leader (5-7 nucleotides) of the βA3/A1 messenger might cause slippage at the first start codon, although the unfavorable context of this start codon might also be responsible. Using transient transfections, we now demonstrate that increasing the length of the leader sequence to 67 nucleotides indeed completely abolishes translation initiation at the second start codon, and thus expression of the βA1-crystallin protein. Messengers having a leader of 5, 7 or 14 nucleotides all express both βA3- and βA1-crystallin at very similar relative levels.

4.2 Introduction

Leaky ribosomal scanning is a mechanism by which multiple length variants of a protein can be produced from a single mRNA through the use of different start codons [1]. This may happen when the first AUG codon is too close to the CAP site or lies in an unfavorable context. The ribosomal complex then occasionally "slips" past this start codon and translation initiation may occur at the second or even at a later start codon. Kozak [2] has found the optimal consensus sequence for translation initiation in higher eukaryotes to be $GCC\begin{smallmatrix}A\\G\end{smallmatrix}CCAUGG$. Start codons can roughly be divided into 'strong' and 'weak' ones [3]. This division is mainly determined by positions -3 and +4 in the consensus sequence, where the A of the start codon is designated +1. Strong start codons are those that either have an A at position -3, or a G at positions -3 and +4. Weak start codons include all other combinations at these positions, and may lead to leaky ribosomal scanning. When the first start codon is strong, leaky ribosomal scanning is unlikely, unless this codon follows within approximately 12 nucleotides after the CAP site [1].

Leaky ribosomal scanning, although not uncommon for viral mRNAs, is extremely rare in eukaryotes. In those rare cases, the resulting proteins always have different functional properties [4-12]. Two members of the β-crystallin family of vertebrate eye lens proteins, βA3 and βA1, have also been demonstrated to be produced from a single βA3/A1 mRNA by

leaky ribosomal scanning [13-16]. Since the two start codons lie in the same reading frame, the two proteins are identical except that βA1 lacks the first 17 amino acids of the βA3 N-terminal extension. There is indeed evidence that βA3- and βA1-crystallin are both required for the subtle interplay of protein repulsion and attraction that warrants lens transparency [17]. Moreover, the evolutionary conservation of the expression of both βA3 and βA1, from chicken [16] to man [15], and possibly in frogs as well [18], also strongly suggests a functional advantage for this phenomenon. Although never actually demonstrated, it has been proposed that leaky ribosomal scanning of the βA3/A1 messenger occurs because of the very short 5' untranslated region of this mRNA [13-16]. The 5' UTR has a length of only five nucleotides in species as diverse as chicken [16], bovine [13, 14], mouse [16] and rat (K.J. Lampi & L.L. David, unpubl.; acc. nr. AF013248). In humans, the length of this sequence was reported to be seven nucleotides [15]. In addition, however, in most species the first start codon can be categorized as 'weak', whereas the second one is 'strong'. In this paper we therefore determine whether it is the length of the 5' UTR of the mammalian βA3/A1-crystallin mRNA that indeed controls the relative expression levels of βA3 and βA1.

4.3 Methods

Construction of eukaryotic expression vectors

To express βA3/A1-crystallin in eukaryotic cells, we obtained the pBSN3Luc vector, which contains the firefly luciferase gene under control of the mouse hsp70.1 promoter [19, 20]. We removed the luciferase gene via *HindIII* and *BamHI* digestion. The 5' overhang was filled in using the Klenow enzyme (Gibco BRL). The vector was circularized by ligation with T4 DNA ligase (Promega), giving pBSN3. In three different mutagenesis reactions (QuikChange Mutagenesis Kit, Stratagene), an *NdeI* site was introduced at different positions after the reported transcription start point (Fig. 1). A bovine/rat chimeric βA3/A1 cDNA was removed from pBSβA3 [21] by *NdeI* and *SacI* digestion. This fragment was then inserted into the three mutated pBSN3 vectors, also digested beforehand with *NdeI* and *SacI*. Correct insertion of the βA3/A1 cDNA into these three vectors was verified by automated sequencing (ALF Express, Pharmacia). Based upon the number of nucleotides present between the reported transcription start point and the first start codon of the βA3/A1 cDNA (contained within the *NdeI* site), thus defining the length of the 5' UTR of the resulting mRNA, these vectors were called pBSNβ-5, pBSNβ-12 and pBSNβ-65, respectively (Fig. 1).

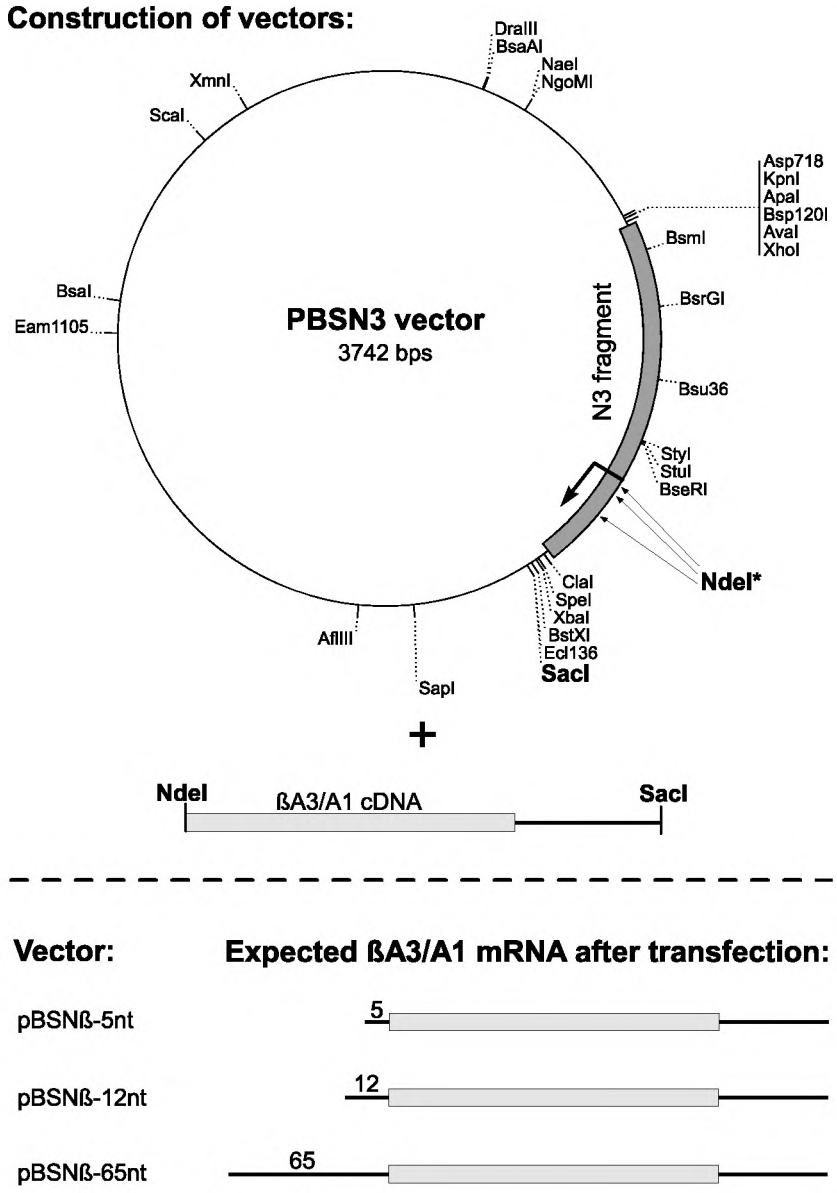


Figure 1. Schematic representation of the cloning strategy for construction of the eukaryotic expression vectors pBSNβ-5, -12 and -65. The βA3/A1 cDNA was ligated between the *SacI* and either of the three introduced *NdeI* sites.

Transformation and cell culture

Chinese hamster ovary cells (CHO-HA1) were cultured under standard conditions in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and antibiotics. For transfections, $3 \cdot 10^5$ cells were seeded into 35-mm dishes. The next day, cells were transfected with 2 µg vector DNA using LipofectAMINE and OptiMEM (Gibco BRL) according to the manufacturer's protocol. One day after transfection, cells were trypsinized and seeded into 100-mm dishes. Forty-eight h after transfection, transcription of the βA3/A1 gene was induced by a heat shock of 15 min at 44°C, applied by immersion of the culture dishes into a

precision water bath (± 0.1°C). Cells were harvested 4 h and 16 h after heating for RNA isolation and immunoblotting, respectively.

Confirmation of the transcription start point

To confirm that we were indeed producing mRNAs of the intended lengths, we had to verify that the reported transcription start point is actually used after transfection of these vectors into CHO cells. For this purpose, total RNA was isolated 4 h after heat shock from a 100-mm culture dish containing CHO cells transfected with pBSNβ-5, using the TRIzol Reagent (Gibco BRL) and the protocol supplied by the manufacturer. The βA3/A1 mRNA was reverse transcribed using a βA3/A1 gene-specific primer, βA3-SP1X (TCTTCATGGAACCC-ACTTCATTGTTG), and the 5' RACE protocol of the 5'/3' RACE Kit (Boehringer Mannheim). After amplification with a second gene-specific primer, βA3-SP2X (GGGTAGTCATCACAGATCTCCCACTGG) and an oligo-dT-anchor primer from the Kit, the resulting cDNA was cloned into the pGEM-T vector (Promega) and sequenced to reveal the length of the 5' leader of the βA3/A1 mRNA in these transfected cells.

Gel electrophoresis and immunoblotting

Samples were loaded on standard 13% SDS polyacrylamide gels and electrophoresed in the Mini Protean II system (Biorad). Gels were blotted onto nitrocellulose membrane (Hybond-C pure, Amersham). βA3 and βA1 were detected using a 1:10,000 dilution of a polyclonal antiserum raised against βA3 in rabbits. This antiserum is able to specifically recognize both βA3- and βA1-crystallin [17, 21]. Alkaline phosphatase conjugated antibodies directed against rabbit IgG were used for staining.

4.4 Results and Discussion

Construction of expression vectors and verification of the transcription start point

To investigate whether the length of the 5' untranslated region of the mammalian βA3/A1 mRNA influences the mechanism of leaky ribosomal scanning that produces βA3- and βA1-crystallin from this single messenger, we intended to construct vectors producing βA3/A1 mRNAs having 5' UTRs of 5, 12 and 65 nucleotides. These lengths were chosen, because (1) almost all investigated βA3/A1 messengers have a 5' UTR of 5 nucleotides, (2) it was reported by Kozak [1] that the process of leaky ribosomal scanning may begin to occur when

A.

```
pBSNβ-5          ...gcagggcggcgagcagggcaccagaccatATGGAGACCCAGACTGTGCAG...
expected mRNA                              accauAUGGAGACCCAGACUGUGCAG...
5'-RACE          ...ttttttttttttttttttttttagaccatATGGAGACCCAGACTGTGCAG...
actual mRNA                              agaccauAUGGAGACCCAGACUGUGCAG...
```

B.

pBSNβ-7 agaccau

pBSNβ-14 agacgcugaaccau

pBSNβ-67 agacgcugacagcuacucagaaucaaaucugguuccauccagagacaagcgaagacaagagaaccau

Figure 2. Determination of the length of the βA3/A1 mRNA leader by RT-PCR after transfection of pBSNβ-5 in CHO cells.

the 5' UTR is around 12 nucleotides, and (3) a 5' leader as long as 65 nucleotides was expected to be sufficiently long for this process not to occur. The βA3/A1 cDNA was therefore ligated into the pBSN3 expression vector, such that the first start codon was 5, 12 or 65 nucleotides downstream of the expected transcription start point. These vectors were transfected into CHO cells to determine the ratio of synthesized βA3- and βA1-crystallin after lysis, SDS-PAGE and Western blotting.

However, before being able to draw any conclusions from the transfection experiments, it was necessary to verify the length of the 5' UTR of the βA3/A1 mRNAs after transfection of our expression constructs. To that end, we performed a 5' RACE with βA3/A1-specific primers on total RNA isolated from CHO cells transfected with pBSNβ-5. The obtained sequence of the 5' terminus of the βA3/A1 mRNA present in these cells revealed a 5' UTR of seven nucleotides rather than the expected five nucleotides (Fig. 2). The sequence of these nucleotides was consistent with a transcription start point starting two positions upstream of the reported transcription start point of the murine hsp70.1 promoter [22] used in the pBSN3 vector. Since the location of this transcription start point was solely inferred from an alignment with the human hsp70 promoter sequence, we must conclude that our 5' RACE experiment has established the actual transcription start point of the murine hsp70.1 promoter. This means, therefore, that the 5' UTRs of our βA3/A1 mRNAs do not have the intended lengths of 5, 12 and 65 nucleotides, but rather are 7, 14 and 67 nucleotides long, respectively. To facilitate readability of this paper, we renamed the constructs pBSNβ-5, -12 and -65, as detailed in Methods, to pBSNβ-7, -14 and -67 in the following section of the paper.

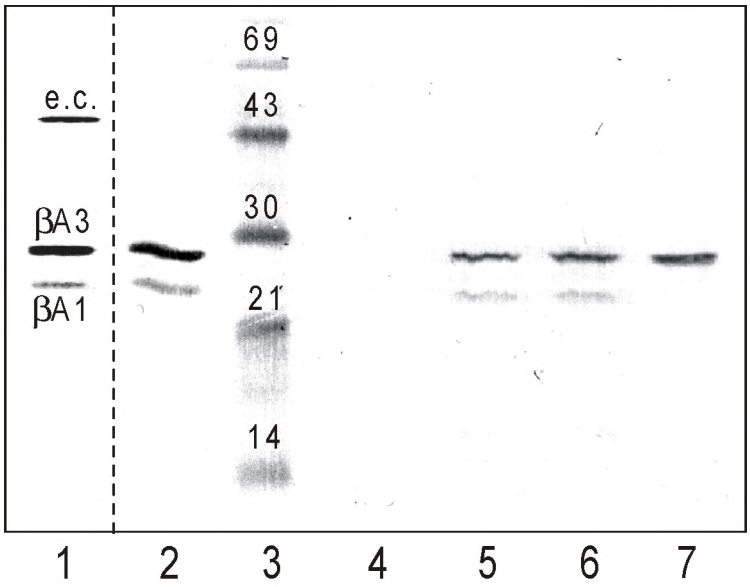


Figure 3. Relative expression levels of βA3- and βA1-crystallin after transfection of pBSNβ-7, -14 and -67 in CHO cells. Lane 1: autoradiograph of an *in vitro* translation of isolated calf lens βA3/A1 mRNA in rabbit reticulocyte lysate (taken from [14]). The abbreviation "e.c." stands for the endogenous component of the rabbit reticulocyte lysate translation system. Lane 2: Western blot of differentiated rat lens epithelium explants. Lane 3: pre-stained molecular weight markers (SeeBlue/Novex; mass in kDa). Lane 4: Western blot of untransfected CHO cells. Lanes 5, 6 and 7: Western blot of CHO cells transfected with pBSNβ-7, pBSNβ-14 and pBSNβ-67, respectively. The Western blot was stained using a polyclonal antiserum directed against βA3/A1 [17,21].

βA3- and βA1-crystallin expression levels

The relative expression levels of βA3 and βA1 in CHO cells transfected with pBSNβ-7, pBSNβ-14 and pBSNβ-67 are shown in Figure 3. It appears that for pBSNβ-7 and -14 (lanes 5 en 6) the ratios of βA3:βA1 are identical to those found in an *in vitro* cell-free expression system using isolated calf lens βA3/A1 mRNA (lane 1; [14]) and to those found in a rat lens epithelium explant system (lane 2; for a description of the explant system, see [23]). The latter two systems both express the wild-type βA3/A1 mRNA with a 5' UTR of only 5 nucleotides. The expression pattern of βA3 and βA1 in CHO cells containing the pBSNβ-67 vector is very different, however, since only βA3 and no βA1 expression could be detected in these transfected cells (Fig. 3, lane 7). Apparently, with a 5' UTR of 67 nucleotides leaky ribosomal scanning of the βA3/A1 messenger does not occur, leading us to the conclusion that length is indeed the determining factor in this case.

One might argue that the unfavorable context of the first start codon could also be important to promote leaky ribosomal scanning for the βA3/A1 messenger. However, in contrast to mammalian βA3/A1 mRNAs, which have an unfavorable pyrimidine at position -3 relative to the first start codon, the chicken messenger has a G at position -3 in combination

	↷ βA3	↷ βA1
Chicken	augacAUGggc	gcaaagAUGgcu . . .
Mouse	accagAUGgag	accaagAUGgcu . . .
Rat	accagAUGgag	accaagAUGgcu . . .
Cow	uccagAUGgag	accaagAUGgcu . . .
Human	guaccagAUGgag	accaagAUGgcu . . .

Figure 4. Alignment showing the context of the two start codons on βA3/A1-crystallin mRNAs from different species.

with a G at position +4 (Fig. 4). This makes it a 'strong' translation initiation site [3]. We have found, nevertheless, that chicken eye lenses still contain the βA1 protein and that its relative amount compared to βA3 is certainly not less than in eye lenses of other species studied in our laboratory (data not shown). This is in agreement with *in vitro* cell-free translation experiments performed on hybridization-selected chicken ßA3/A1 mRNA, where βA1 and βA3 are expressed in almost similar amounts [16]. These results clearly argue against the importance of the context of the first start codon in directing leaky ribosomal scanning of the βA3/A1 messenger.

We conclude, therefore, that the shortness of the 5' leader sequence of the βA3/A1 messenger is indeed the sole cause of leaky ribosomal scanning normally occurring for this messenger. Increasing the length of the leader of this messenger to well over 60 nucleotides completely abolishes the production of βA1-crystallin. During evolution, the 5' and 3' UTRs of mRNAs are in general much less conserved in length and sequence than are the coding regions [24]. The fact that the 5' UTR of the βA3/A1 mRNA has retained its unusual shortness for at least 310 million years, since birds and mammals diverged [25], thus strongly supports the notion that the balance between βA3- and βA1-crystallin is indispensable for proper eye lens functioning [17].

4.5 Acknowledgments

We thank Dr. Gloria Li of the Memorial Sloan-Kettering Cancer Center in New York for her kind donation of the pBSN3Luc vector, and Dr. Azem Civil of the Department of Molecular Biology of the University of Nijmegen for his help with the rat lens epithelium explants.

4.6 References

1. Kozak M (1991) J. Cell Biol. 115: 887-903
2. Kozak M (1987) Nucleic Acids Res. 15: 8125-8148
3. Kozak M (1989) J. Cell Biol. 108: 229-241
4. Shaper NL, Hollis GF, Douglas JG, Kirsch IR & Shaper JH (1988) J. Biol. Chem. 263: 10420-10428
5. Tuboi S, Suzuki T, Sato M & Yoshida T (1990) Adv. Enzyme Regul. 30: 289-304
6. Descombes P & Schibler U (1991) Cell 67: 569-579
7. Slusher LB, Gillman EC, Martin NC & Hopper AK (1991) Proc. Natl. Acad. Sci. USA 88: 9789-9793
8. Pietrini G, Aggujaro D, Carrera P, Malyszko J, Vitale A & Borgese N (1992) J. Cell Biol. 117: 975-986
9. Lin FT, MacDougald OA, Diehl AM & Lane MD (1993) Proc. Natl. Acad. Sci. USA 90: 9606-9610
10. Ossipow V, Descombes P & Schibler U (1993) Proc. Natl. Acad. Sci. USA 90: 8219-8223
11. Tenhunen J & Ulmanen I (1993) Biochem. J. 296: 595-600
12. Spotts GD, Patel SV, Xiao Q & Hann SR (1997) Mol. Cell. Biol. 17: 1459-1468
13. Gorin MB & Horwitz J (1984) Curr. Eye Res. 3: 939-948
14. Quax-Jeuken Y, Janssen C, Quax W, van den Heuvel R & Bloemendal H (1984) J. Mol. Biol. 180: 457-472
15. Hogg D, Tsui LC, Gorin M & Breitman ML (1986) J. Biol. Chem. 261: 12420-12427
16. Peterson CA & Piatigorsky J (1986) Gene 45: 139-147
17. Werten PJL, Vos E & de Jong WW (1999) Exp. Eye Res. (in press)
18. Lu SF, Pan FM & Chiou SH (1996) Biochem. Biophys. Res. Commun. 221: 219-228.
19. Kim D, Ouyang H, Yang SH, Nussenzweig A, Burgman P & Li GC (1995) J. Biol. Chem. 270: 15277-15284.
20. Li GC & Nussenzweig A (1996) EXS 77: 425-449
21. Werten PJL, Carver JA, Jaenicke R & de Jong WW (1996) Protein Eng. 9: 1021-1028
22. Hunt C & Calderwood S (1990) Gene 87: 199-204
23. Chamberlain CG & McAvoy JW (1989) Growth Factors 1: 125-134
24. Li W-H & Graur D (1991) Fundamentals of molecular evolution. Sinauer Associates, Sunderland, Massachusetts.
25. Kumar S & Hedges SB (1998) Nature 392: 917-920

CHAPTER V

Formation of βA3/βB2-crystallin mixed complexes: involvement of N- and C-terminal extensions

Paul J.L. Werten, Robyn A. Lindner, John A. Carver & Wilfried W. de Jong

Biochimica et Biophysica Acta **1432**, 286-292.

5.1 Abstract

The sequence extensions of the β-crystallin subunits have been suggested to play an important role in the oligomerization of these eye lens proteins. This, in turn, may contribute to maintaining lens transparency and proper light refraction. In homo-dimers of the βA3- and βB2-crystallin subunits, these extensions have been shown by ^{1}H-NMR spectroscopy to be solvent-exposed and highly flexible. In this study, we show that βA3- and βB2-crystallins spontaneously form mixed βA3/βB2-crystallin complexes, which, from analytical ultracentrifugation experiments, are dimeric at low concentrations (< 1 mg/ml) and tetrameric at higher protein concentrations. ^{1}H-NMR spectroscopy reveals that in the βA3/βB2-crystallin tetramer, the N-terminal extensions of βA3-crystallin remain water-exposed and flexible, whereas both N- and C-terminal extensions of βB2-crystallin lose their flexibility. We conclude that both extensions of βB2-crystallin are involved in protein-protein interactions in the βA3/βB2-crystallin hetero-tetramer. The extensions may stabilize and perhaps promote the formation of this mixed complex.

5.2 Introduction

The vertebrate eye lens contains high concentrations of water-soluble proteins named crystallins. The three major types, α-, β- and γ-crystallin, can be found in eye lenses of practically all vertebrates. α-Crystallin is a member of the small heat-shock protein family, whereas the β- and γ-crystallins form a separate protein superfamily. Although quite similar in sequence and structure, γ-crystallins are strictly monomeric, whereas β-crystallins can form dimers and higher oligomeric complexes (for a review, see [1]). Among the seven known mammalian γ-crystallins, only γS-crystallin has a short four amino acid N-terminal extension, which is highly flexible [2,3]. The other γ-crystallins only have a two amino acid, hydrophobic C-terminal extension. In contrast to the γ-crystallins, β-crystallins possess much longer N- and sometimes C-terminal extensions [4]. These extensions have been suggested to play an important role in β-crystallin oligomerization. Some have proposed that the extensions may facilitate β-crystallin oligomerization, by stabilizing protein-protein interactions [5,6,7]. Others have suggested that the extensions may serve as physical spacers, preventing further oligomerization [8], or as a means to regulate repulsive charge between β-crystallin complexes, thus preventing aggregation and maintaining lens transparency [9]. To further explore the role of the β-crystallin extensions, we have studied their behavior in the

βA3/βB2-crystallin hetero-oligomer, which is formed spontaneously upon mixing of the two proteins. ^{1}H-NMR spectroscopy was used to determine whether the conformational flexibility of any of the extensions in this complex was diminished by hetero-oligomerization, which would imply that the particular extension is involved in complex formation.

5.3 Materials and Methods

Purification of proteins

Recombinant βA3-crystallin was expressed in *E. coli* BL21(DE3) and purified as described previously [9]. βB2-crystallin was isolated from bovine lenses using a modification of the procedure of Mostafapour and Schwartz [10]. Briefly, ten calf lenses were homogenized in 20 ml of buffer containing 20 mM NaH_2PO_4/Na_2HPO_4 (pH 6.9), 100 mM Na_2SO_4 and 1 mM EDTA. All insoluble material was removed by centrifugation at 10,000 rpm and 4°C for 45 minutes (Sorvall SA-600). The water-soluble bovine lens proteins were separated on an ACA-34 gel permeation column. Fractions containing βH-, βL1- and βL2-crystallin were pooled in 50 ml plastic tubes, and DTT was added to a final concentration of 1 mM. The tubes were placed in boiling water for 5 minutes. After heating, they were allowed to cool down by placing them on ice for 15 minutes. All β-crystallins denature and precipitate during this procedure, except βB2-crystallin, which is thermostable and refolds to its native state [11]. Denatured proteins were removed by centrifugation at 5000 rpm and 4°C for 15 minutes (Minifuge GL, Heraeus Christ). The supernatant was then subjected to a second round of heating, cooling and centrifugation. The final supernatant, containing pure βB2, was dialyzed against water and lyophilized. 1D and 2D TOCSY ^{1}H-NMR spectra acquired on this sample at approximately 20 mg/ml in 50 mM phosphate buffer, 0.05% NaN_3, pH 7.3 were identical to those for βB2-crystallin isolated by column chromatography [12].

Analytical gel filtration

Hetero-oligomers of βA3- and βB2-crystallin were prepared by mixing equal amounts of both proteins and incubating them for 4 h at 37°C, allowing spontaneous exchange of subunits. The newly formed βA3/βB2-crystallin complexes were then applied to a Superdex-75 PG 16/60 gel filtration column (Pharmacia) to determine their apparent molecular mass. The column was loaded with 2.0 mg of the βA3/βB2-crystallin complex in 1.0 ml 50 mM phosphate buffer at pH 7.0 and eluted at room temperature with the same buffer at a flow rate of 1.0 ml/min. Proteins used to correlate apparent molecular mass with elution volume of the

Superdex-75 column were bovine serum albumin (67 kDa), βB2-crystallin (dimer of 46 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (14 kDa).

Analytical ultracentrifugation

In addition to the gel filtration experiment, analytical ultracentrifugation was used to determine the oligomeric size of the βA3/βB2-crystallin complexes at higher protein concentrations. Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge at 25°C. Samples were centrifuged for up to 30 hours at 17,000 rpm. Double sector cells were used. Two loading concentrations (undiluted, i.e. the same concentration as in the ^{1}H-NMR experiment, and a two-fold dilution) were used in centerpieces of two different widths (1.74 mm for the two-fold dilutions, and 0.78 mm for the undiluted sample). Scans were acquired at 280 and 360 nm, giving absorbance (and thus concentration) versus radius over time. Data were baseline-corrected by subtracting A360 data from A280 data, and corrected for the varying path lengths.

To determine if sedimentation and chemical equilibrium have been attained, the Omega function [13] can be used. The Omega function for a self-associating system is a continuous function of the total concentration of associating solute at any point in the centrifuge and the parameters of the association reaction (i.e. equilibrium constants and second virial coefficients). If sedimentation and chemical equilibrium are attained, the Omega function data collected from the experimental data (over a common concentration range) will superimpose on a single continuous curve. This allows for a test for both the attainment of equilibrium and the absence of contaminants. Fitting of the Omega function with specified reaction models allows for the determination of the parameters of self-association. Molecular mass and omega calculations were performed using the program OMMENU* [14,15]. For ultracentrifuge calculations, an extinction coefficient of 2.5 g^{-1} cm^{-1} (i.e. an average of the values for βA3- and βB2-crystallin) was used. Buffer density was 1 g/l and partial specific volume was 0.73 ml/g.

^{1}H-NMR of βA3/βB2-crystallin complexes

^{1}H-NMR spectra were acquired at 600 MHz and 25°C on a Bruker DMX-600 NMR spectrometer using the methods outlined in Werten *et al.* [9]. All samples were dissolved in 0.7 ml of 50 mM phosphate buffer, 0.05% NaN_3, pH 7.0 in 90% H_2O / 10% D_2O. The

* OMMENU is available via anonymous FTP transfer from the RASMB archive (bbri.harvard.edu)

βA3/βB2-crystallin solution had a protein concentration of approximately 16 mg/ml. Protein concentrations were determined by the Bradford assay [16].

5.4 Results:

Analytical gel filtration

To determine the oligomeric size of βA3/βB2-crystallin, mixed complexes were generated as described in Materials and Methods, and chromatographed over a Superdex-75 column. Care was taken to mix equal amounts of βA3- and βB2-crystallin. Elution time of the sample was correlated to the elution times of proteins of known molecular mass. From Figure 1A it can be seen that the βA3/βB2-crystallin complex elutes as a dimer under these conditions (i.e. an apparent molecular mass of ~ 49 kDa), similar to βA3-crystallin (~ 50 kDa) and somewhat larger than βB2-crystallin (~ 46 kDa). Figure 1B confirms that hetero-dimers are indeed

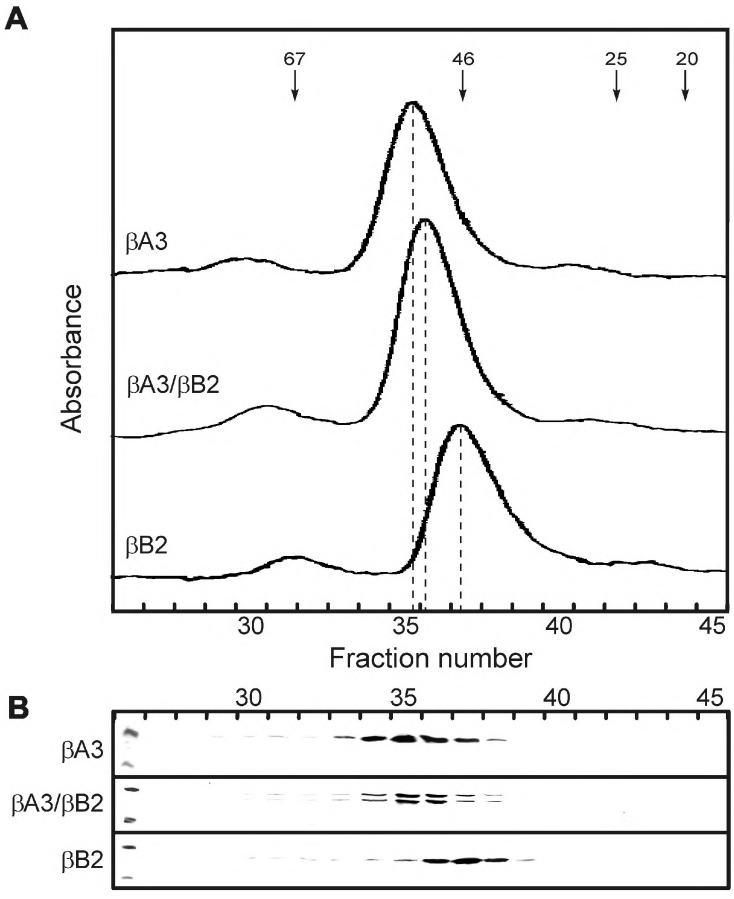


Figure 1. Gel filtration chromatography of βA3-, βA3/βB2-, and βB2-crystallin on a Superdex-75 column. (A) Estimation of the apparent molecular mass of βA3-, βA3/βB2-, and βB2-crystallins by correlation of their elution volumes with molecular mass marker (indicated in kDa by arrows in the top of the figure). The broad peaks eluting at lower fraction numbers in each profile arise from a small amount of tetrameric species. (B) SDS-PAGE analysis of 2 ml fractions of the chromatographs shown above. Perfect co-elution of βA3- and βB2-crystallins in the mixed sample, in contrast to their elution in separate runs, confirms formation of hetero-dimers. Numbers indicate the fractions analyzed.

formed, as recently also observed by Hejtmancik *et al.* [17], since βA3- and βB2-crystallin in the mixed complexes now perfectly co-elute, whereas the individually eluted crystallins homo-dimers are separated by approximately two fractions of 2 ml each.

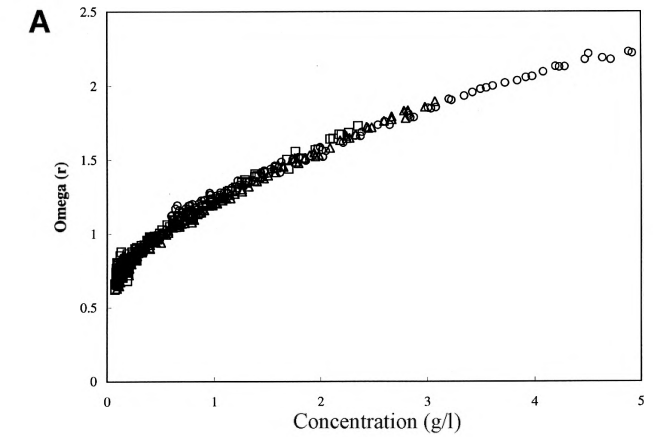
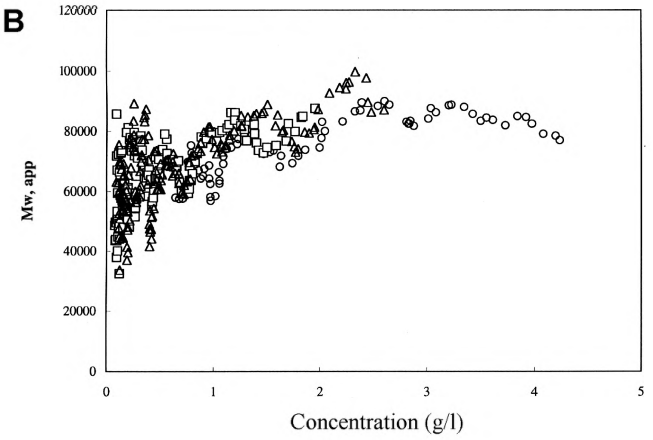
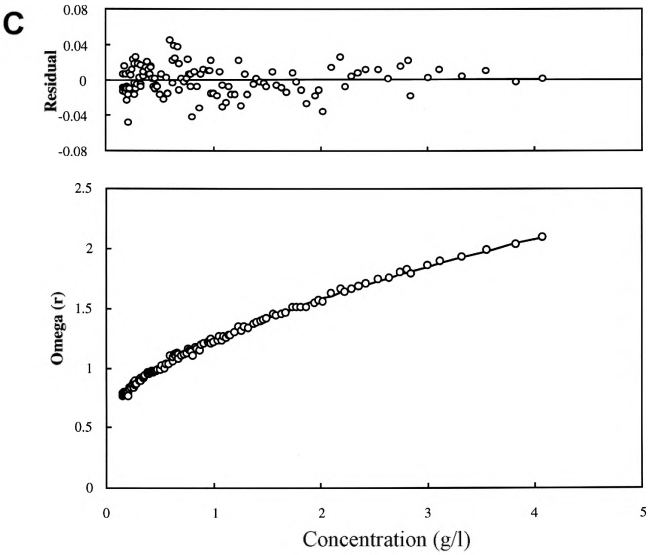


Figure 2. Determination of the complex size of βA3/βB2-crystallin by Analytical Ultracentrifugation.

(A) Overlay of Omega (r) versus concentration for three βA3/βB2-crystallin samples (O = 16 mg/ml; □ , △ = 8 mg/ml). Good overlap of data over a common concentration range is observed, indicating that both chemical and sedimentation equilibrium have occurred.



(B) Apparent mass-average molecular mass versus concentration of βA3/βB2-crystallin complexes. Data converge around 50 kDa at zero concentration, indicating that the predominant low molecular mass species is a dimer. With increasing concentration, the molecular mass approaches that of a tetramer. Symbols are as in (A).



(C) Fit of a two species model (line in lower panel) to data for the undiluted sample, i.e. 16 mg/ml (O). Protomer molecular mass is taken as 50 kDa. The narrow and even distribution of residuals (upper panel) shows that the two species model accurately describes the equilibrium between βA3/βB2-crystallin hetero-dimers and -tetramers.

Analytical ultracentrifugation

As can be seen from Figure 2A, good overlay of the Omega function [13] was obtained for the three samples of βA3/βB2-crystallin complexes (one undiluted sample of 16 mg/ml and two two-fold diluted samples), indicating that both sedimentation and chemical equilibrium had been obtained, and that there were little or no contaminants (suggesting no proteolysis or aggregation problems). The apparent mass-average molecular mass versus concentration plot (Fig. 2B) converges to around 50 kDa at zero concentration, indicating that this is the predominant low molecular mass species. This is consistent with the expected molecular mass of 48 kDa for the βA3/βB2-crystallin hetero-dimer. At the highest concentration, the molecular mass is around 90 kDa. This value is below the expected molecular mass of 96 kDa for the hetero-tetramer due to non-ideality arising from the high concentrations of protein used in these experiments, which will result in a slight downward curvature of molecular mass versus concentration plots at very high protein concentration.

Omega versus concentration data were then fitted with a two species model (Fig. 2C), which gave an excellent fit to the data (as shown by the narrow random residual distribution), with a protomer molecular mass of 50 kDa (indicating a dimer-tetramer reaction) and an equilibrium constant of around 1 l/g for this reaction. The spread of residuals is slightly lerger than usual, as well as the experimental spread observed in Figure 2B. Importantly, however, the random distribution of residuals indicates the absence of disturbing features, such as irreversible aggregation. It can thus be concluded that at low concentrations (e.g. on a gel filtration column; Fig. 1), βA3/βB2-crystallin will be mostly present as a hetero-dimer, but at higher concentrations (such as for the ^{1}H-NMR experiments), βA3/βB2-crystallin will be present as a hetero-tetramer.

^{1}H-NMR spectroscopy of βA3/βB2-crystallin

A solution containing an equimolar mixture of βA3- and βB2-crystallin was prepared. As discussed above, sedimentation equilibrium studies on this solution indicated that, under the experimental conditions used, the protein was a tetramer. Two-dimensional (2D) ^{1}H-NMR spectra were acquired on this βA3/βB2-crystallin mixture to ascertain the effect of hetero-tetramer formation on the conformational flexibility of the terminal extensions of both proteins. The βB2-crystallin homo-dimer has N- and C-terminal extensions whose conformational flexibility is readily apparent from examination of its NMR spectra [12].

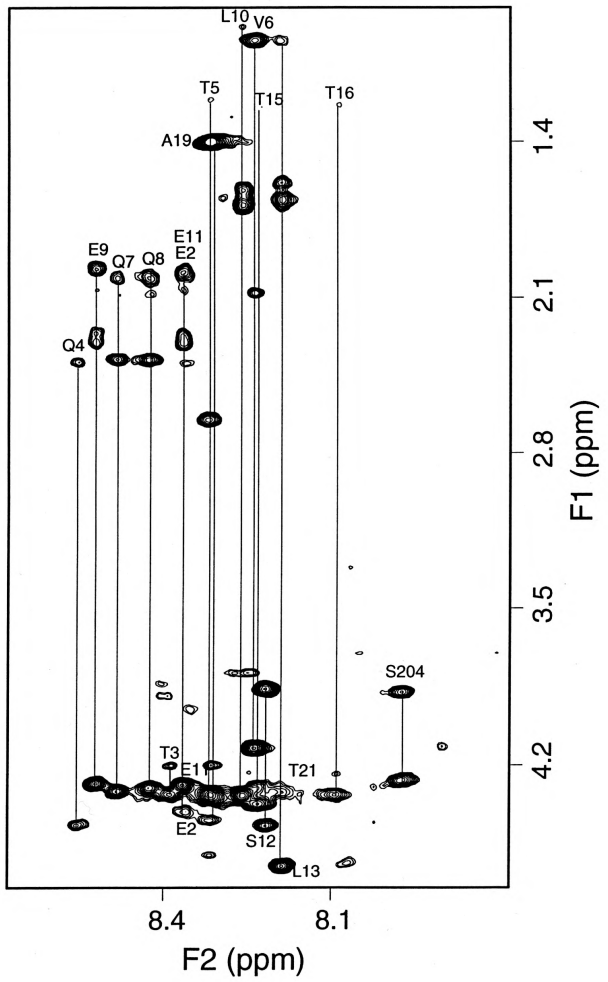


Figure 3. NH to α, β, γ-CH region of the TOCSY spectrum (spin lock mixing time = 70 ms) of βA3/βB2-crystallin. Assignments are indicated for the N-terminal extension of βA3-crystallin and the C-terminal residue (Ser204) in βB2-crystallin (spin lock mixing time of 70 ms). Weak cross-peaks for Asn22 of βA3-crystallin were observed in this region of a TOCSY spectrum acquired with a spin lock mixing time of 30 ms, indicating that this residue has slightly diminished flexibility.

Likewise, in spectra of the βA3-crystallin homo-dimer, the first 22 amino acids in the N-terminal extension are flexible [9]. In 2D NMR spectra of βB2/βA3-crystallin, cross-peaks from the majority of residues in the N-terminal extension of βA3-crystallin (up to Asn22) were well resolved (Fig. 3), i.e. the degree of flexibility observed in the homo-dimer was retained in the hetero-tetramer. By contrast, only cross-peaks from the C-terminal Ser204 of βB2-crystallin were well resolved in spectra of the βA3/βB2-crystallin hetero-tetramer (Fig. 3). The NH resonance of Ser204 has a characteristic upfield chemical shift [12]. The assignment of Ser204 resonances was confirmed in a NOESY spectrum by the presence of the expected NOE between NH proton of Ser204 and the α-CH of Ser203 [12]. Thus upon formation of mixed tetramers, flexibility of the N-terminal extension of βA3-crystallin has

been maintained, but the N- and C-terminal extensions of βB2-crystallin have almost totally lost their flexibility.

5.5 Discussion:

In this study we have shown that βA3- and βB2-crystallin can spontaneously form mixed complexes, in agreement with previous results by Hejtmancik *et al.* [17]. In contrast to their partial (~50%) hetero-complex formation after 4-6 h at room temperature, our current results indicate that hetero-complex formation after a 4 h incubation at 37°C is complete. This difference may be due to the fact that higher protein concentrations and a higher temperature were used during our recombination experiments. The ability of different β-crystallins to spontaneously and completely form mixed complexes has also been described for βB2 and βB3 [18].

At low concentrations, βA3/βB2-crystallin complexes form hetero-dimers, as shown by analytical gel filtration, but these dimers can further oligomerize to form hetero-tetramers at higher protein concentrations, as shown by analytical ultracentrifugation. This is in agreement with the observation by Slingsby *et al.* [19], who, using gel filtration at high concentration, have shown that tetramers of βA3/βB2-crystallin had formed after mixing of denatured βA3- and βB2-crystallin and renaturation.

Upon formation of βA3/βB2-crystallin hetero-tetramers, both the N- and C-terminal extensions of βB2, which are flexible in the homo-dimer, can no longer be detected by ^{1}H-NMR spectroscopy, indicating that they are involved in protein-protein interactions in the βA3/βB2-crystallin complex. We propose that interactions of βB2-crystallin extensions with βA3 stabilize the βA3/βB2-crystallin heteromeric complex and may actually serve to promote formation of this specific complex. This idea is consistent with the observation that βA3/βB2-crystallin is the major component of the tetrameric β-crystallin fraction in lens extract [19]. Seemingly in contrast with this result, flexibility was observed to a varying extent for both βB2-crystallin extensions in NMR spectra of the various β-crystallin aggregates isolated from calf lens extracts by size exclusion chromatography [20]. The N-terminal extension was observed in spectra of all β-crystallin aggregates, but the C-terminal extension loses its flexibility in the higher molecular mass aggregate, βH-crystallin (which corresponds approximately to the octameric species). It should be noted, however, that these fractions contain a wide variety of hetero-oligomers composed of the seven different β-crystallin subunits in the calf lens. Therefore, conclusions regarding the flexibility of the βB2-crystallin

extensions in those complexes cannot be extrapolated to mixtures of individual β-crystallin subunits, such as the βA3/βB2-crystallin tetramer. It does indicate, however, that in lens extracts there must be complexes containing βB2-crystallin in which its extensions are not involved in protein-protein interactions.

Based on modeling of a theoretical βA4/βB2-crystallin hetero-dimer to the known 3D structure of βB2-crystallin (Fig. 4 in [1]), it is tempting to speculate that the C-terminal extension of βB2-crystallin, which is in the vicinity of the N-terminal domain of its acidic partner, could bind to this domain. Such an interaction may indeed exist in the βA3/βB2-crystallin hetero-dimer and could specifically add to the inter-domain interactions that most likely exist between all β-crystallin subunits. As for the model of a theoretical hetero-tetramer (Fig. 5B in [1]), the N-terminal extension of the basic β-crystallin subunit in the foreground dimer can be seen coming close to the C-terminal domain of the acidic subunit in the background dimer, and *vice versa*. If these extensions are indeed able to bind there, this could mean that they may serve to stabilize the dimer-dimer interaction. These assumptions, applied to the βA3/βB2-crystallin hetero-oligomers, would predict the absence of resonances of both N- and C-terminal extensions of βB2-crystallin in the hetero-tetramer, in agreement with the ^{1}H-NMR spectra of βA3/βB2-crystallin, and the absence of only the C-terminal extension of βB2-crystallin in the hetero-dimer. Unfortunately, the protein concentration of a solution containing only hetero-dimers of βA3/βB2-crystallin would be too low to verify this by ^{1}H-NMR spectroscopy.

It would seem, therefore, that the N- and C-terminal extensions of the β-crystallins can have functions that may differ for each individual β-crystallin subunit, and even for the particular complexes these subunits are present in. Our results clearly show that both extensions of βB2-crystallin are involved in protein-protein interactions in the βA3/βB2-crystallin hetero-tetramer, whereas in other β-crystallin complexes they are sometimes free [20]. In contrast, the N-terminal extensions of βA3-crystallin in the βA3/βB2-crystallin hetero-tetramer remain solvent exposed and may act as physical spacers [8] or as carriers of repulsive charge [9], preventing protein aggregation in the lens, and thus maintaining lens transparency.

Further NMR experiments with various combinations of β-crystallin subunits must be undertaken to shed some light on the complicated process of β-crystallin complex formation and the interactions between these complexes that help determine the physical properties of the lens.

5.6 Acknowledgments

We wish to thank Dr. Christine Slingsby for useful discussions regarding β-crystallin structure and models. This work was supported by EC-HCM grant CHRX-CT93-0175 (to W.W.deJ.) and by grant 980497 from the National Health and Medical Research Council of Australia (to J.A.C.).

5.7 References:

[1] C. Slingsby, B. Norledge, A. Simpson, O.A. Bateman, G. Wright, H.P.C. Driessen, P.F. Lindley, D.S. Moss, B. Bax, X-ray diffraction and structure of crystallins, Prog. Ret. Eye Res. 16 (1997) 3-29.

[2] Y. Quax-Jeuken, H. Driessen, J. Leunissen, W. Quax, W.W. de Jong, H. Bloemendal, βS-Crystallin: structure and evolution of a distinct member of the βγ-superfamily, EMBO J. 4 (1985) 2597-2602.

[3] P.G. Cooper, J.A. Carver, J.A. Aquilina, G.B. Ralston, R.J.W. Truscott, A ^{1}H-NMR spectroscopic comparison of γS- and γB-crystallins, Exp. Eye Res. 59 (1994) 211-220.

[4] N.H. Lubsen, H.J.M. Aarts, J.G.G. Schoenmakers, The evolution of lenticular proteins: the β- and γ-crystallin super gene family, Prog. Biophys. Molec. Biol. 51 (1988) 47-76.

[5] G.A. Berbers, A.M. Brans, W.A. Hoekman, C. Slingsby, H. Bloemendal, W.W. de Jong, Aggregation behavior of the bovine β-crystallin Bp chain studied by limited proteolysis, Biochim. Biophys. Acta 748 (1983) 213-219.

[6] J.N. Hope, H.C. Chen, J.F. Hejtmancik, βA3/A1-Crystallin Association - Role of the N-Terminal Arm, Protein Eng., 7 (1994) 445-451.

[7] Y. Sergeev, P. Wingfield, J. Hope, J.F. Hejtmancik, The role of C-terminal extension in βB2-crystallin oligomerization, Invest. Ophtalmol. Vis. Sci. 38 (1997) S206.

[8] S. Trinkl, R. Glockshuber, R. Jaenicke, Dimerization of βB2-crystallin: The role of the linker peptide and the N- and C-terminal extensions, Protein Sci. 3 (1994) 1392-1400.

[9] P.J.L. Werten, J.A. Carver, R. Jaenicke, W.W. de Jong, The elusive role of the N-terminal extension of βA3- and βA1-crystallin, Protein Eng. 9 (1996) 1021-1028.

[10] M.K. Mostafapour, C.A. Schwartz, Purification of a heat-stable beta-crystallin polypeptide of the bovine lens, Curr. Eye Res. 1 (1981) 517-522.

[11] J. Horwitz, M. McFall-Ngai, L-L Ding, O. Yaron, Thermal stability of lens crystallins, in: G. Duncan (ed.), Topics in aging research in Europe, vol. 6, The lens: transparency and cataract, EURAGE, Rijswijk, the Netherlands, 1986, pp. 227-240.

[12] J.A. Carver, P.G. Cooper, R.J.W. Truscott, ^{1}H-NMR spectroscopy of βB2-crystallin from bovine lens. Conformation of the N- and C-terminal extensions, Eur. J. Biochem. 213 (1993) 313-320.

[13] B.K. Milthorpe, P.D. Jeffrey, L.W. Nichol, The direct analysis of sedimentation equilibrium results obtained with polymerising systems, Biophys. Chem. 3 (1975) 169-176.

[14] M. Morris, G.B. Ralston, Determination of the parameters of self-association by direct fitting of the omega function, Biophys. Chem. 23 (1985) 49-61.

[15] G.B. Ralston, and M. Morris, in: S.E. Harding, A.J. Rowe, J. C. Horton (Eds.), Analytical Ultracentrifugation in Biochemistry and Polymer Science, Royal Soc. Chem., Cambridge, 1992, pp. 253-274.

[16] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[17] J.F. Hejtmancik, P.T. Wingfield, C. Chambers, P. Russell, H.C. Chen, Y.V. Sergeev, J.N. Hope, Association properties of βB2- and βA3-crystallin: ability to form dimers, Protein Eng. 10 (1997) 1347-1352.

[18] C. Slingsby, O.A. Bateman, Formation and crystallization of the eye lens heterodimer βB2-βB3-crystallin, Exp. Eye Res. 58 (1994) 761-764.

[19] C. Slingsby, O.A. Bateman, Quaternary interactions in eye lens β-crystallins: basic and acidic subunits of β-crystallin favor heterologous association, Biochemistry 28 (1990) 6593-6599.

[20] P.G. Cooper, J.A. Carver, R.J.W. Truscott, ^{1}H-NMR spectroscopy of bovine lens β-crystallin. The role of the βB2-crystallin C-terminal extension in aggregation, Eur. J. Biochem. 213 (1993) 321-328.

CHAPTER VI

Gecko iota-crystallin: how cellular retinol-binding protein became an eye lens ultraviolet filter

Paul J.L. Werten, Beate Röll, Daan M.F. van Aalten & Wilfried W. de Jong

Proceedings of the National Academy of Science of the USA, **97**, in press.

6.1 Abstract

Eye lenses of various diurnal geckos contain up to 12% of iota-crystallin. This protein is related to cellular retinol-binding protein type I (CRBP I), but has 3,4-didehydroretinol, rather than retinol, as a ligand. The 3,4-didehydroretinol gives the lens a yellow color, thus protecting the retina by absorbing short-wave radiation. Iota-crystallin could be either the gecko's housekeeping CRBP I, recruited for an additional function in the lens, or the specialized product of a duplicated CRBP I gene. The finding of the same CRBP I-like sequence in lens and liver cDNA of the gecko *Lygodactylus picturatus* now supports the former option. Comparison with iota-crystallin of a distantly related gecko, *Gonatodes vittatus*, and with mammalian CRBP I, suggests that acquiring the additional lens function is associated with increased amino acid changes. Compared with the rat CRBP I structure, the iota-crystallin model shows reduced negative surface charge, which might facilitate the required tight protein packing in the lens. Other changes may provide increased stability, advantageous for a long-living lens protein, without frustrating its role as retinol transporter outside the lens. Despite a number of replacements in the ligand pocket, recombinant iota-crystallin binds 3,4-didehydroretinol and retinol with similar and high affinity (~1.6 nM). Availability of ligand thus determines whether it binds 3,4-didehydroretinol, as in the lens, or retinol, in other tissues. Iota-crystallin presents a striking example of exploiting the potential of an existing gene, without prior duplication.

6.2 Introduction

To function properly, the vertebrate eye lens must contain high concentrations of water-soluble proteins, the crystallins [1,2]. In most vertebrates, the bulk of the lens proteins is formed by the β/γ-crystallins, a superfamily of stable proteins of obscure evolutionary origins. Large quantities of αA- and αB-crystallin are generally also present in the lens. These belong to the small heat-shock protein superfamily and may function as molecular chaperones to prevent aggregation of proteins in the lens. In addition to these ubiquitous crystallins, many species also express high levels of one or more of the so-called taxon-specific crystallins. All but one of the 11 characterized taxon-specific crystallins are identical or closely related to regular house-keeping enzymes, mostly oxidoreductases such as lactate dehydrogenase B and α-enolase [1-6]. Some of these enzyme crystallins may have been recruited for their additional function in the lens because of the associated high levels of pyridine nucleotide cofactors, which may confer protection against oxidative damage [2,7].

The only non-enzyme taxon-specific crystallin observed thus far is iota-crystallin [6], which is present at levels ranging from 2 to 12% in certain species of diurnal geckos [8]. A partial protein sequence of this monomeric 16-kDa lens protein showed that it belongs to the family of intracellular lipid-binding proteins (iLBPs), being most closely related to cellular retinol-binding protein type I (CRBP I). This protein is ubiquitous and is involved in retinoid storage and transport [9,10]. However, although retinol is the normal ligand for CRBP I, iota-crystallin from gecko lens contains 3,4-didehydroretinol (vitamin A_2) as a ligand. The 3,4-didehydroretinol · iota-crystallin complex gives the lenses of these geckos a yellow coloring and absorbs short-wave radiation. This characteristic improves the optical quality of the lens, and provides an excellent protection against ultraviolet damage to the retina. The latter seems particularly useful for diurnal geckos, which have no eyelids and cannot regulate the aperture of their pupil and live in habitats where they are exposed to high intensities of light [6].

The expression in gecko lenses of a CRBP I-like protein with the unusual ligand 3,4-didehydroretinol raises important evolutionary questions. First of all, is iota-crystallin the product of a duplicated CRBP I gene, allowing specific adaptations for functioning as a lens protein? Or rather, could iota-crystallin be the gecko's housekeeping CRBP I, over-expressed in the lens to gain a dual function? Second, has the structure of iota-crystallin been adapted to harbor 3,4-didehydroretinol as a ligand, and does it have other properties that might support its functioning in the lens? Finally, can the evolutionary pathways that led to the recruitment of iota-crystallin in various genera of geckos be reconstructed? To answer these questions, we determined the cDNA sequences for eye lens iota-crystallin and for its housekeeping homologue in the liver of the gecko *Lygodactylus picturatus*, and found them to be identical. The obtained DNA sequence was used to reconstruct the phylogenetic relationship of iota-crystallin with other known CRBPs. A partial protein sequence of iota-crystallin from a distantly related gecko, *Gonatodes vittatus*, was obtained to reconstruct the evolutionary history of this protein more accurately. A three-dimensional iota-crystallin model was constructed to understand its ligand-binding and structural properties, and recombinant iota-crystallin of *L. picturatus* was produced to study its retinoid-binding behavior.

6.3 Materials and Methods

Cloning of iota-crystallin

Total RNA was isolated from three lenses of *L. picturatus,* using the TRIzol reagent (Gibco/BRL), and cDNA was synthesized with the 5'/3' rapid amplification of cDNA ends kit (Roche Molecular Biochemicals). Amplification was performed with a primer based on the partial protein sequence of iota-crystallin (ref. 6; ATGCCNCCNAAYTTYACNGG,

corresponding with positions 1-6 in Fig. 1 and including the start codon) and an anchor primer from the rapid amplification of cDNA ends kit. The resulting cDNA was cloned into the pGEM-T vector (Promega) to give iota/pGEM-T, and three independent clones were sequenced (ALF Express, Amersham Pharmacia).

Cloning of gecko CRBP I

Isolation of RNA from two *L. picturatus* livers, and cDNA synthesis were performed as described above. A first amplification was performed with a primer based on nucleotide sequences of human, rat and mouse CRBP I and iota-crystallin (CTGAAGCCRGACAAR-GAGATC, corresponding to positions 36-42 in Fig. 1) and an anchor primer from the rapid amplification of cDNA ends kit. This PCR product was the template for another round of amplification with the primer ACCAYATGAYCATCCGCAC (corresponding to positions 47-53 in Fig. 1) and the anchor primer. The resulting cDNA was cloned and sequenced as described above.

Protein sequence of Gonatodes iota-crystallin

Water-soluble proteins from *G. vittatus* eye lenses were separated by SDS/PAGE, blotted onto PVDF membrane (Immobilon-P, Millipore), stained with Coomassie blue and partially destained. The band corresponding to iota-crystallin, migrating at 16 kDa, was excised and further destained. Amino acid sequencing was performed on a Hewlett-Packard G1000A protein sequencer.

Modeling

A structural model for *L. picturatus* iota-crystallin was made by homology modeling (WHATIF; ref. 11), with rat CRBP I (PDB entry 1CRB; ref. 12) as the template. Rotamers of conserved residues were left unchanged. All other residues were mutated initially to alanines, and rotamers were modeled with the WHATIF backbone-dependent rotamer libraries [13]. The resulting model was energy-minimized by using the steepest descents algorithm in the GROMOS87 programs [14]. Calculations were performed *in vacuo* with crystallographic waters by using the GROMOS reduced charges forcefield. All calculations included positional restraints on the Cα atoms to the template coordinates (force constant 9,000 $kJ \cdot mol^{-1} \cdot nm^{-2}$) and 3,4-didehydroretinol was included in the calculations with a modified GROMOS87 retinol topology. After energy minimization, 5 ps of molecular dynamics at 300 K was performed to relax the structure further, followed by conjugate gradient energy minimization until no significant energy difference could be observed. Analysis of

electrostatic surface potential was performed by using GRASP [15], and other pictures were made with Bobscript [16] and Raster3D [17].

Expression and purification of recombinant iota-crystallin

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to introduce an *NdeI* site (containing the initiation codon ATG of iota-crystallin) in the vector iota/pGEM-T. This vector, which already contains an *NdeI* site downstream of the iota-crystallin sequence, was digested with *NdeI.* The 665-bp fragment containing the iota-crystallin cDNA was isolated from a 1% agarose gel with the QiaEx II Gel Extraction Kit (QiaGen, Chatsworth, CA) and cloned into a pET3a vector for transfection into *E. coli* BL21(DE3). Protein expression was induced by isopropyl-β-D-thiogalactoside. Water-soluble lysate was produced [18] in phosphate buffer (4 mM Na_2HPO_4, 4 mM KH_2PO_4, 14 mM KCl, 0.1 mM EDTA, 20 mg/l NaN_3, 3 mg/l DTT, 500 mg/l PMSF, pH 6.8), and 25-ml fractions were applied to a DEAE-Sepharose Fast Flow column (50 ml; Amersham Pharmacia). Iota-crystallin was eluted with a second phosphate buffer (6.6 mM Na_2HPO_4, 6.6 mM KH_2PO_4, 23.3 mM KCl, 0.166 mM EDTA, 33.3 mg/l NaN_3, 5 mg/l DTT, pH 6.8), whereas almost all *E. coli* proteins remained bound. Suitable fractions were concentrated (Nova 3K disc membrane; Filtron, Karlstein, Germany) and purified further over Superdex-75 PG 16/60 (Amersham Pharmacia). Peak fractions of iota-crystallin were stored at 4°C for further use. Purity was assessed by SDS/PAGE, and identification was performed by Western blotting with a monoclonal antibody against bovine CRBP I [8].

Fluorescence assays for retinoid-binding

The precise concentration of active iota-crystallin was determined by fluorescence titration with all-*trans*-retinol as described [19], except that retinol instead of protein fluorescence was measured. Stoichiometric binding of ligand was ensured by using protein concentrations far above the dissociation constant for retinol · CRBP I [19,20]. Fluorescence was measured at 458 nm (bandpass 20 nm) with excitation at 348 nm (bandpass 3 nm) in the absence of light on a Shimadzu RF-5301PC spectro-fluorophotometer. Stock retinoid solutions in ethanol were prepared in the dark, and concentrations determined on a Perkin Elmer lambda-15 spectrometer with the absorption coefficients: all-*trans*-retinol, $\varepsilon = 46{,}000\ M^{-1}\cdot cm^{-1}$ at 325 nm [21]; all-*trans*-retinoic acid, $\varepsilon = 45{,}000\ M^{-1}\cdot cm^{-1}$ at 350 nm [21] ; all-*trans*-3,4-didehydro-retinol, $\varepsilon = 41{,}300\ M^{-1}\cdot cm^{-1}$ at 350 nm [22]. For direct binding assays, increasing amounts of all-*trans*-retinol were added to 1.5 ml of a 20 nM solution of iota-crystallin in phosphate

buffer. Fluorescence was measured twice on two samples for each data point. The increase in retinol fluorescence was plotted as a function of retinol concentration, and the dissociation constant of the retinol · iota-crystallin complex was calculated by nonlinear least-squares fitting [19]. For competition binding assays, increasing amounts of either all-*trans*-3,4-didehydroretinol or all-*trans*-retinoic acid were added to 1.5 ml of a 250 nM solution of iota-crystallin and a saturating amount (350 nM) of all-*trans*-retinol. This iota-crystallin concentration is well above the value of the dissociation constant of the retinol · iota-crystallin complex. Fluorescence was measured twice on a single sample for each data point. All samples were allowed to equilibrate for at least 15 min at room temperature in the dark before measurement. The final amount of ethanol in all samples never exceeded 1%.

6.4 Results

Sequence characteristics of L. picturatus iota-crystallin

During the initial characterization of iota-crystallin, as isolated from the eye lenses of *L. picturatus*, the N-terminal sequence up to position 83 was determined by automated Edman degradation [6]. To deduce the complete amino acid sequence, we now performed reverse transcription-PCR on total RNA from *L. picturatus* lenses with a primer designed on the basis of the very N-terminal protein sequence of iota-crystallin. This procedure provided the sequence of the coding region, from nucleotide position 21 onward, and the 149 nucleotides of the 3' untranslated region of iota-crystallin cDNA. The deduced amino acid sequence is 134 residues in length, giving a calculated molecular mass of 15,788 Da. Alignment with all available sequences of CRBP types I and II, which are known only for mammals, confirms that the protein is most closely related with CRBP I, with 59-60% sequence identity (Fig. 1). As compared with rat CRBP I, the number of charged residues is reduced in iota-crystallin: Asp+Glu from 25 to 21 and Lys+Arg from 19 to 17. An extra proline in iota-crystallin, next to the N-terminal proline which is also present in CRBP I, is of interest. According to the "N-end rule" [23], an N-terminal proline may increase the average lifetime of a protein by protecting against degradation. A second proline might enhance this effect by providing a secondary barrier. There also is a trend to replace smaller with larger aliphatic residues: Ala+Val from 14 to 9 and Leu+Ile from 18 to 25. Proteins from thermophilic bacteria indicate a correlation between thermostability and a so-called aliphaticity index, calculated from the relative volume occupied by aliphatic residues [24]. Iota-crystallin has a higher aliphaticity index (83.58) than that of rat CRBP I (77.01), which suggests a somewhat increased stability.

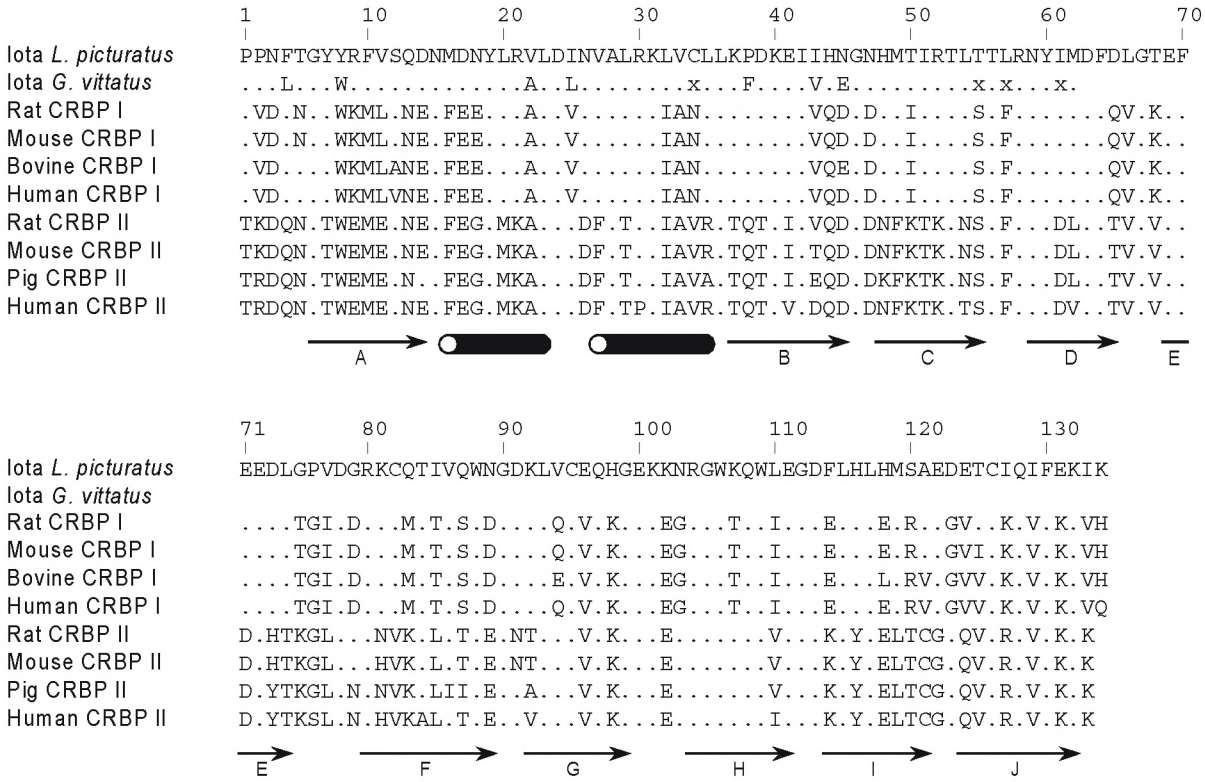


Figure 1. Iota-crystallin sequences aligned with mammalian CRBP I and II. The topmost sequence, from position 7 onward, is deduced from the iota-crystallin mRNA of *L. picturatus*. It corrects the previously reported incomplete sequence [6] at positions 64-67, 69 and 79. The second sequence is the partial sequence of *G. vittatus* iota-crystallin, as determined by Edman degradation. Residues that are identical to the topmost sequence are indicated by dots. Positions of β-strands A-J and two α-helices in the CRBP structure [12] are indicated. Accession numbers for CRBP I are G809309 for rat, Q00915 for mouse, AAB34275 for cow and P09455 for human; accession numbers for CRBP II are A92065 for rat, Q08652 for mouse, P50121 for pig and AAC50162 for human.

Iota-crystallin is the gecko CRBP I

If iota-crystallin were the gecko housekeeping CRBP I, upregulated in the lens, one would expect that CRBP I-like proteins in other tissues are identical to iota-crystallin. On the other hand, if iota-crystallin were the product of a duplicated CRBP I gene, the genuine housekeeping CRBP I should be found in other organs, possibly together with iota-crystallin. Reverse transcription-PCR was therefore performed on total RNA from liver, an organ with high CRBP I levels [9,10]. Two CRBP I-specific primers, designed on the basis of conserved regions in mammalian CRBP I and iota-crystallin cDNA sequences, were used in consecutive PCRs. These PCRs yielded a single cDNA sequence, identical to the last 266 nucleotides of the coding sequence and the entire 3' untranslated region of the iota-crystallin mRNA as isolated from the lens. The fact that the only CRBP I-like gene product detected in gecko liver is identical with iota-crystallin indicates that it is indeed the housekeeping CRBP I that is upregulated as a crystallin in the lens. Iota-crystallin/CRBP I is thus a convincing example of

"gene sharing", a phenomenon in which a protein is recruited to serve an additional function without loosing its original one [3,25,26].

Evolution of iota-crystallin

Western blotting has demonstrated the presence of iota-crystallin/CRBP I-like proteins in lens extracts from diurnal geckos belonging to two other Old World genera, *Quedenfeldtia* and *Pristurus*, and to the New World genus *Gonatodes* [8]. The CRBP I-like lens protein of *G. vittatus* deviates from that of the Old World genera by having a slightly larger apparent molecular mass and by having both 11-*cis* and all-*trans* 3,4-didehydroretinol as natural ligands [8]. In lenses of the Old World geckos only the all-*trans* form is found. Because of these differences, we performed automatic Edman degradation on *Gonatodes* iota-crystallin, isolated by SDS/PAGE and blotting onto PVDF membrane. This procedure identified 58 residues in the N-terminal sequence, of which 51 are identical to *Lygodactylus* iota-crystallin (Fig. 1). Over this sequence the *Gonatodes* and *Lygodactylus* iota-crystallins are approximately equidistant to mammalian CRBP I (66% and 64% sequence identity, respectively). This finding indicates that the *G. vittatus* protein is the orthologue of *L. picturatus* iota-crystallin.

The data set of Fig. 1 was used to obtain an impression of the relationship of iota-crystallin to mammalian CRBP I and II. Fig. 2 presents the tree that was obtained by neighbor-joining analysis [27] of the protein alignment. As expected, iota-crystallins are closest to CRBP I. Interestingly, the branch lengths indicate a considerably faster rate of change at the protein level in the gecko iota-crystallin/CRBP I lineage, because it diverged

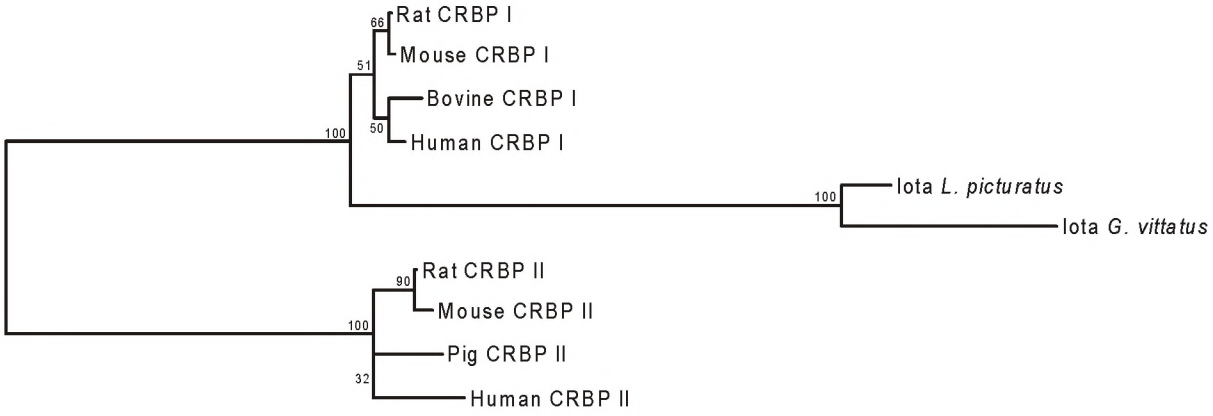


Figure 2. Iota-crystallins in the phylogenetic tree of the CRBPs. This neighbor-joining tree [27] is constructed from the aligned protein sequences in Fig. 1 by using a Tajima-Nei distance matrix [28] and midpoint rooting. Unassigned positions in *Gonatodes* iota-crystallin are introduced as missing data. Bootstrap scores are based on 1,000 replicates. Branch lengths are proportional to the number of amino acid replacements.

from the lineage towards mammalian CRBP I. This faster rate of change may in part reflect the accumulation of selectively advantageous amino acid changes enabling the additional function of CRBP I as a lens protein in geckos.

Structural model of iota-crystallin

One wonders whether such adaptive changes can be observed when the iota-crystallin sequence is modeled by using rat CRBP I as the template structure. CRBP I has, like the other iLBPs, a β-barrel structure made up of 10 antiparallel strands, with the ligand localized inside the barrel [10,29]. In agreement with the amino acid composition of iota-crystallin, analysis of electrostatic surface potential shows a reduction of -- mostly negative -- surface charge, especially at the front (strands A-F; Fig. 3). Reduction of negative surface charge reduces

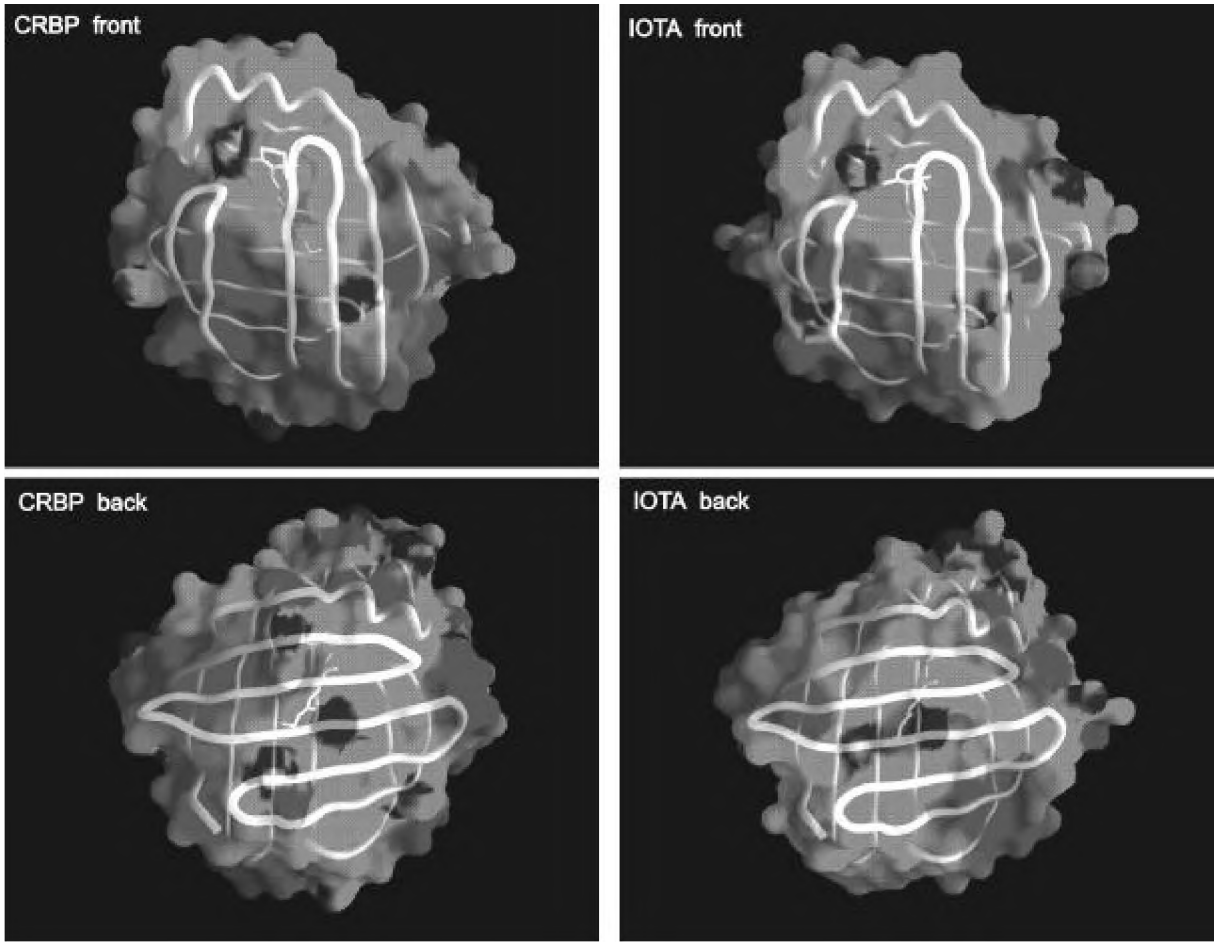


Figure 3. Reduced surface charge of iota-crystallin (Right) as compared with rat CRBP I (Left). (Upper) Front view. (Lower) Back view. Molecular surfaces, calculated in GRASP [15], are colored according to electrostatic potential: red < -5.0 kT; blue > 5 kT. The Cα backbone worm is shown in white, and retinoids are in a stick representation (3,4-didehydroretinol for iota-crystallin; retinol for CRBP I).

For full color images see original paper or optional color inlay in this thesis.

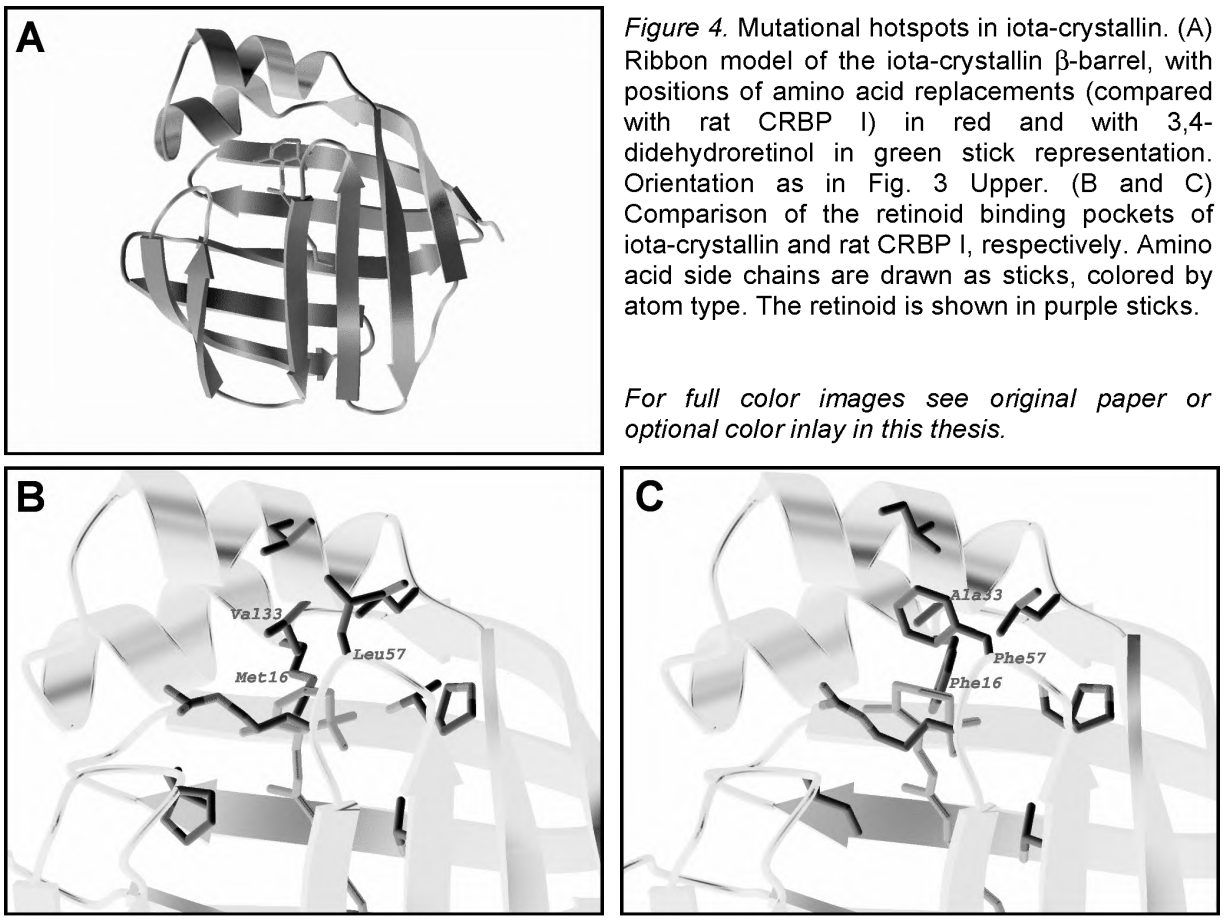


Figure 4. Mutational hotspots in iota-crystallin. (A) Ribbon model of the iota-crystallin β-barrel, with positions of amino acid replacements (compared with rat CRBP I) in red and with 3,4-didehydroretinol in green stick representation. Orientation as in Fig. 3 Upper. (B and C) Comparison of the retinoid binding pockets of iota-crystallin and rat CRBP I, respectively. Amino acid side chains are drawn as sticks, colored by atom type. The retinoid is shown in purple sticks.

For full color images see original paper or optional color inlay in this thesis.

repulsive behavior, thereby allowing a protein to achieve high concentrations, in line with the close packing of proteins needed in the eye lens to obtain a high refractive index [30]. The mutations in iota-crystallin are concentrated in a few areas (Fig. 4A). In strands F-J, forming the β-sheet behind the retinoid, strings of residues running perpendicular to the strand directions, have been mutated. All these residues point towards the solvent. The residues lining the pocket of the retinoid tail, i.e., pointing inward on the two β-sheets, have been completely conserved. However, there is a cluster of mutations around the retinoid ring, on the tips of the C-D and E-F turns, and on the inside of the helices. These areas are compared for iota-crystallin and rat CRBP I in Figs. 4B and 4C. The residue that is in closest contact with the retinoid is at position 33 (3.7 Å for both iota-crystallin and CRBP I). This residue is an alanine in mammalian CRBP I and II but a valine in iota-crystallin, both in *Lygodactylus* and *Gonatodes*. Further mutations around the retinoid ring are Phe-16 → Met and Phe-57 → Leu. These three mutations could represent adaptive modifications to optimize the fit for 3,4-didehydroretinol in iota-crystallin. Alternatively, they may equally well be selectively neutral changes.

Ligand-binding properties of recombinant iota-crystallin

To assess the actual retinoid-binding properties of iota-crystallin, the recombinant *L. picturatus* protein was expressed in *E. coli* and purified. A solution containing a precisely determined concentration of active iota-crystallin was used in a direct binding assay with all-*trans*-retinol. Retinol fluorescence was measured and plotted as a function of total retinol concentration (Fig. 5A). Non-linear least-squares fitting of the data gave K_d = 1.606 nM and n = 1.043 as best estimates (R^2 = 0.997) for the apparent dissociation constant of the all-*trans*-retinol · iota-crystallin complex and the correction factor for the iota-crystallin concentration, respectively. This dissociation constant is similar to values published for bovine CRBP I [19], although higher values have also been reported [20]. The ability of all-*trans*-3,4-didehydroretinol to compete with all-*trans*-retinol for binding to iota-crystallin is shown in Fig. 5B. The binding curve of all-*trans*-3,4-didehydroretinol drops to half the fluorescence intensity [i.e., ½ · (F_0 + F_∞)] at a molar ratio to all-*trans*-retinol equal to one. This value indicates that iota-crystallin has similar and high affinity for both of these retinoids. Fig. 5B also shows that all-*trans*-retinoic acid, another physiologically relevant retinoid, is unable to compete with all-*trans*-retinol. These observations are comparable to those reported for rat CRBP I, which also has similar affinities for all-*trans*-retinol and all-*trans*-3,4-didehydroretinol, and where all-*trans*-retinoic acid is unable to compete for binding [20].

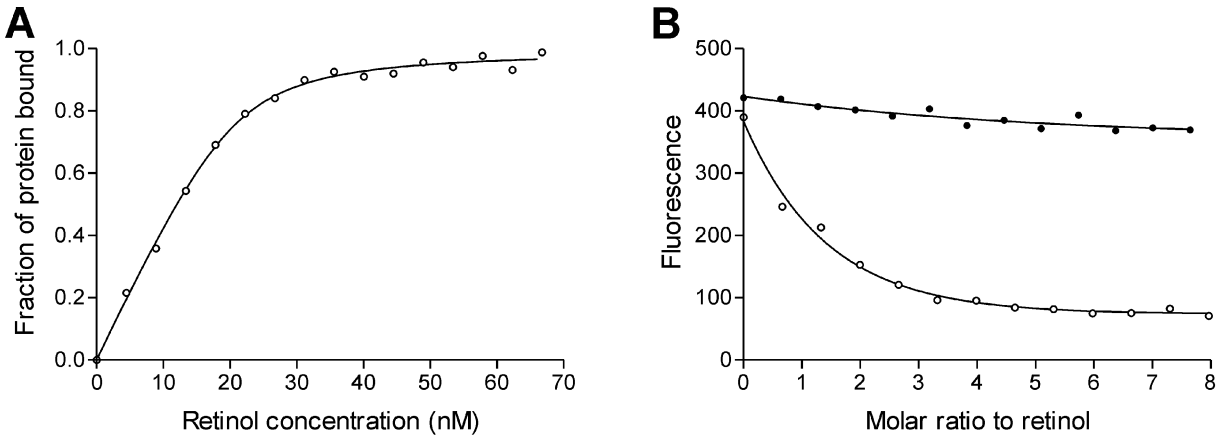


Figure 5. Ligand-binding of recombinant iota-crystallin. (A) Determination of the dissociation constant of iota-crystallin for all-*trans*-retinol. Open circles indicate the fraction of liganded iota-crystallin at increasing total retinol concentrations, as calculated from measured retinol fluorescence. The continuous line is the nonlinear least-squares fitting of these data, giving a K_d of 1.606 nM. (B) Competition binding assays, presenting remaining retinol fluorescence after competitive binding of all-*trans*-retinoic acid (closed circles) and all-*trans*-3,4-didehydroretinol (open circles).

6.5 Discussion

Our results suggest the following scenario for the evolutionary origin of gecko lens iota-crystallin. The mostly nocturnal gecko family is supposed to have descended from a diurnal lizard ancestor [31]. Several genera of geckos have reverted to diurnal habits, often living in environments where they are exposed to intense ambient light. The eyelids of these geckos are fused to form a transparent spectacle, and they are unable to regulate incident light by changing the pupil diameter. Consequently, the only means to avoid that potentially harmful radiation, especially UV and short-wave blue light, reaches the retina is to absorb it in the lens. This absorption can be accomplished by the accumulation of suitable lens pigments. Diurnal geckos of the genera *Lygodactylus*, *Gonatodes*, *Quedenfeldtia* and *Pristurus* use 3,4-didehydroretinol, bound to iota-crystallin/CRBP I, for this purpose [6,8]. This situation required complex evolutionary processes that had to occur in concert to have any adaptive value. Although not normally occurring in the lens, one can imagine that CRBP I became expressed in the gecko lens, initially at low levels, by chance mutations in the promoter of its gene. Interestingly, another iLBP, related to epidermal fatty acid binding protein, has been found at low levels in the bovine lens [32]. It may be relevant in this respect that iLBP genes have the potential to become highly expressed, given the presence of 18% muscle fatty acid-binding protein in adult locust flight muscle [33]. Interesting, too, is the discovery that *O*-crystallin from the octopus eye lens is related to the phosphatidylethanolamine-binding proteins, another family of lipid-binding proteins [34].

A gradually increasing expression of CRBP I in the lens would probably be selectively neutral, because CRBP I, like other iLBPs, is a very stable protein [29]; as such, it is acceptable as a lens protein. However, retinol, the natural ligand of CRBP I, is unsuitable as a lens chromophore, because it is not photostable and fluoresces in the range of visible light [35]. In contrast, 3,4-didehydroretinol is photostable and does not fluoresce but is rarely found in terrestrial vertebrates. Vitamin A_2-based visual pigments do in fact occur in some lizards [36], but both nocturnal and diurnal geckos use retinol for visual pigment generation [6]. However, in the eye cups of diurnal *Lygodactylus* geckos, but not in those of nocturnal geckos, 3,4-didehydroretinol and its ester are found [8]. It therefore seems that the synthesis of a suitable lens chromophore, as a ligand for iota-crystallin required the activation of an enzyme that converts retinol into 3,4-didehydroretinol specifically for this purpose. Because this conversion apparently occurs in the retinal pigment epithelium [8], the chromophore must be transported into the lens -- by ways that are as yet unknown -- before it can be liganded to iota-crystallin. Considering the complexity of this suite of events it seems most likely that it occurred only once in some ancestral gecko lineage. This does not necessarily imply that the

presence of iota-crystallin in four gecko genera indicates their monophyly; the expression of iota-crystallin may have faded again in certain daughter lineages, as has been proposed for other taxon-specific crystallins [1,2].

Acquiring the additional function as a lens protein, while maintaining its role as retinol transporter in other tissues, may have imposed specific demands on gecko iota-crystallin/CRBP I. In the lens, iota-crystallin/CRBP I should bind its chromophore without the need to release it again, and an increased stability would be favorable for a long-living lens protein. To function as a retinol transporter, iota-crystallin/CRBP I should be able to bind retinol, and release it again as needed [37], thus requiring a certain degree of structural flexibility [38]. Like mammalian CRBP I [20], iota-crystallin binds all-*trans*-3,4-didehydroretinol equally as well as all-*trans*-retinol (Fig. 5B). Because 3,4-didehydroretinol is the only retinoid present in the gecko eye lens [8], there is no real requirement for specific adaptations of iota-crystallin to obtain preferential binding of this chromophore. Indeed, although our modeling studies identified replacements in the binding pocket of iota-crystallin around the retinoid ring, suggesting that it is optimized for all-*trans* 3,4-didehydroretinol, we could not demonstrate any higher affinity in our retinoid-binding assays. Furthermore, any optimization in this direction would almost certainly affect the retinol transporter functions of iota-crystallin in non-lens tissues. Ruling out this option, therefore, it seems that iota-crystallin/CRBP I has sustained the following adaptive changes to make it a better lens protein. First, trends in amino acid substitutions and the proline at position 2 lead to increased stability and lowered turnover. Second, diminished negative surface charge reduces repulsive interactions, thereby facilitating close packing of proteins, in line with the need for a high refractive index of the eye lens [30]. The apparently increased rate of change in the iota-crystallin lineage after its divergence from the lineage to mammalian CRBP I may in part reflect these adaptive trends.

During evolution, various other chromophores have been recruited in vertebrate lenses to reduce the deleterious effects of short-wave radiation. The yellowish lenses of human, squirrel, and some fishes contain kynurenine derivatives [39,40], whereas mycosporine-like amino acids occur in the lenses of many marine fishes [40]. Lipid-soluble carotenoids also serve this purpose and are bound to the lens protein α-crystallin in the hatchetfish *Argyropelecus affinis* [41] and to γs-crystallin in the gecko *Quedenfeldtia trachyblepharus* [42]. The recruitment of CRBP I as a lens protein, and the concomitant synthesis of a stable and nonfluorescent chromophore further indicate the versatility of organisms to cope with novel environmental demands and present a convincing example of adaptive evolution at the molecular level.

6.6 Acknowledgements

We thank Dr. Willem de Grip and Petra Geurts for help with the retinoid-binding experiments, Ole Madsen for phylogenetic tree constructions, and Hoffmann-La Roche for the gift of vitamin A_2.

References

1. de Jong, W.W., Lubsen, N.H. & Kraft, H.J. (1994) *Progr. Retin. Eye Res.* **13**, 391-442.

2. Wistow, G. (1995) *Molecular biology and evolution of crystallins: gene recruitment and multifunctional proteins in the eye lens.* (Landes, Austin, TX).

3. Piatigorsky, J. & Wistow, G. (1989) *Cell* **57**, 197-199.

4. Jimenez-Asensio, J., Gonzalez, P., Zigler, J.S., Jr. & Garland, D.L. (1995) *Biochem. Biophys. Res. Commun.* **209**, 796-802.

5. Röll, B., Van Boekel, M.A., Amons, R. & de Jong, W.W. (1995) *Biochem. Biophys. Res. Commun.* **217**, 452-458.

6. Röll, B., Amons, R. & de Jong, W.W. (1996) *J. Biol. Chem.* **271**, 10437-10440.

7. Zigler, J.S., Jr. & Rao, P.V. (1991) *FASEB J.* **5**, 223-225.

8. Röll, B. & Schwemer, J. (1999) *J. Comp. Physiol. A* **185**, 51-58.

9. Saari, J.C. (1990) *Prog. Retin. Res.* **9**, 363-381.

10. Newcomer, M.E. (1995) *FASEB J.* **9**, 229-239.

11. Vriend, G. (1990) *J. Mol. Graphics* **8**, 52-56.

12. Cowan, C.W., Newcomer, M.E. & Jones, T.A. (1993) *J. Mol. Biol.* 230, 442-446.

13. Vriend, G. & Sander, C. (1993) *J. Appl. Crystallogr.* **26**, 47-60.

14. van Gunsteren, W.F. & Berendsen, H.C.J. (1987) *Gromos manual*, BIOMOS, Biomolecular Software (Laboratory of Physical Chemistry, Univ. of Groningen, Groningen, The Netherlands).

15. Nicholls, A., Sharp, K. & Honig, B. (1991) *Proteins* **11**, 281-296.

16. Esnouf, R.M. (1997) *J. Mol. Graphics* **15**, 132-134.

17. Bacon, D. & Anderson, W.F. (1988) *J. Mol. Graphics* **6**, 219-220.

18. Werten, P.J.L., Carver, J.A., Jaenicke, R. & de Jong, W.W. (1996) *Protein Eng.* **9**, 1021-1028.

19. Malpeli, G., Stoppini, M., Zapponi, M.C., Folli, C. & Berni, R. (1995) *Eur. J. Biochem.* **229**, 486-493.

20. MacDonald, P.N. & Ong, D.E. (1987) *J. Biol. Chem.* **262**, 10550-10556.

21. Horwitz, J. & Heller, J. (1973) *J. Biol. Chem.* **248**, 6317-6324.

22. von Planta, C., Schweitzer, U., Chopard-dit-Jean, L., Ruegg, R., Kofler, M. & Isler, O. (1962) *Helv. Chim. Acta* **45**, 548-561.

23. Gonda, D.K., Bachmair, A., Wunning, I., Tobias, J.W., Lane, W.S. & Vashavsky, A. (1989) *J. Biol. Chem.* **264**, 16700-16712.

24. Ikai, A. (1980) *J. Biochem.* **88**, 1895-1898.

25. Piatigorsky, J., O'Brien, W.E., Norman, B.L., Kalumuck, K., Wistow, G.J., Borras, T., Nickerson, J.M. & Wawrousek, E.F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3479-3483.

26. Hendriks, W., Mulders, J.W., Bibby, M.A., Slingsby, C., Bloemendal, H. & de Jong, W.W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7114-7118.

27. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406-425.

28. Tajima, F. & Nei, M. (1984) *Mol. Biol. Evol.* **1**, 269-285.

29. Banaszak, L., Winter, N., Xu, Z., Bernlohr, D.A., Cowan, S. & Jones, T.A. (1994) *Adv. Protein Chem.* **45**, 89-151.

30. Tardieu, A., Vérétout, F., Krop, B. & Slingsby, C. (1992) *Eur. Biophys. J.* **21**, 1-12.

31. Walls, G.L. (1942) *The vertebrate eye and its adaptive radiation* (Cranbrook Inst. Sci., Bloomfield Hills, MI).

32. Jaworski, C.J. & Wistow, G.J. (1994) *Invest. Ophthalmol. Visual Sci.* **35**, 1706.

33. Haunerland, N.H., Chen, X., Andolfatto, P., Chisholm, J.M. & Wang, Z. (1993) *Mol. Cell. Biochem.* **123**, 153-158.

34. Zinovieva, R.D., Piatigorsky, J. & Tomarev, S.I. (1999) *Biochim. Biophys. Acta* **1431**, 512-517.

35. Ong, D.E. & Chytil, F. (1978) *J. Biol. Chem.* **253**, 828-832.

36. Provencio, I., Loew, E.R. & Foster, R.G. (1992) *Vision Res.* **32**, 2201-2208.

37. van Aalten, D.M.F., Findlay, J.B.C., Amadei, A. & Berendsen, H.J.C. (1995) *Protein Eng.* **8**, 1129-1135.

38. Jamison, R.S., Newcomer, M.E. & Ong, D.E. (1994) *Biochemistry* **33**, 2873-2879.

39. Zigman, S. & Paxhia, T. (1988) *Exp. Eye Res.* **47**, 819-824.

40. Thorpe, A., Douglas, R.H. & Truscott, R.J. (1993) *Vision Res.* **33**, 289-300.

41. Yu, N.T., Cai, M.Z., Lee, B.S., Kuck, J.F., Jr., McFall-Ngai, M. & Horwitz, J. (1991) *Exp. Eye Res.* **52**, 475-479.

42. Röll, B. (2000) *Comp. Biochem. Physiol.*, in press.

CHAPTER VII

General Discussion

7.1 Evolution of the crystallins

As has become evident in the previous Chapters, the crystallin proteins of the eye lens bear evidence of a whole range of molecular mechanisms that have been successfully applied by evolution. As indicated in Chapter I, there seems to be a general stress connection in the acquisition of both the ubiquitous α-, β- and γ-crystallins and the taxon-specific crystallins. This can be explained by the requirements placed on candidate lens proteins by evolution, where those proteins are favored that are water-soluble, stable and long-lasting, and that could moreover protect the structure and content of the transparent lens-fiber cells from UV and oxidative damage.

In general, the acquisition of proteins as structural lens proteins has either happened by using the same gene product for both the lens and non-lens function -- a process that is called gene sharing [Piatigorsky & Wistow, 1989] -- or by duplicating the gene of the candidate protein and adapting one copy for its task in the lens. Both of these events have certainly played a role in the evolution of the α-crystallins [De Jong *et al.*, 1989]. αB-Crystallin, which is the oldest of the two α-crystallins and was a small heat-shock protein in several tissues -- most likely including the lens -- before αA existed, got up-regulated in the lens to function as a structural lens protein. After that, a gene duplication most likely allowed the development of αA-crystallin. The latter can be considered as much more lens-specific and was no doubt optimized as a lens protein in ways that were not achievable for αB-crystallin, which still had to fulfill its function as a heat-shock protein in other tissues.

More gene duplication events have taken place during the evolution of the β/γ-crystallin superfamily. Here, a process called gene fusion was used in addition to a number of gene duplications to build a two domain/four motif protein out of a one domain/one motif ancestral protein (see Chapter I). The split in β- and γ-crystallins early on in this process, gave evolution the opportunity to experiment with different properties and configurations of these proteins. All seven mammalian γ-crystallins known today -- again products of several gene duplications of the ancestral γ-crystallin gene -- are strictly monomeric proteins [Lubsen *et al.*, 1988]. The short-range non-specific interactions between the γ-crystallins are overall attractive, allowing dense packing of these proteins [Tardieu *et al.*, 1992]. High relative levels of γ-crystallins have therefore been considered responsible for the high refraction index and hard nature of rodent and fish eye lenses [de Jong, 1981]. In the β-crystallins another level of diversification is present. Apart from the fact that, here as well, gene duplications have led to seven β-crystallin proteins that exist in two groups (acidic and basic β-crystallins) [Lubsen *et*

al., 1988], these proteins can form oligomers ranging from dimers to octamers of various mixed compositions [Slingsby & Bateman, 1990]. Overall the β-crystallins show repulsive behavior [Tardieu *et al.*, 1992], contributing to lens transparency and ensuring a much softer nature of eye lenses such as found in most mammals and in birds.

Gene duplication events seem to have the evolutionary advantage that it is subsequently possible to change the properties of one molecule without losing the beneficial effects of the already existing molecule, thus giving evolution much more freedom to experiment. When these proteins are then combined, as is the case for the β-crystallins that oligomerize into mixed complexes of various sizes, the total repertoire can be greatly expanded. Fine-tuning of the expression levels and of the specific and non-specific interactions of the β-crystallins may very well directly help defining the physical properties of eye lens.

It seems that the recruitment of taxon-specific crystallins is a further option to extend the repertoire of proteins in the eye lens, which in turn may enhance or extend some of the eye lens' functions. As can be judged from the number of taxon-specific proteins (see Table I, Chapter I), this option -- may it have been selectively neutral or advantageous -- has been realized by evolution quite a few times. The most dramatic example where extended functionality has indeed been achieved by recruitment of a taxon-specific crystallin is no doubt found in the eye lenses of some diurnal geckos in the form of iota-crystallin, which will be discussed later in this Chapter.

7.2 βA3/A1-crystallin

With the proteins βA3 and βA1, evolution employed another way to further extend the β-crystallin family. βA3- and βA1-crystallin are coded by one gene and translated from a single βA3/A1 messenger [Goring & Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Hogg *et al.*, 1986; Peterson & Piatigorsky, 1986]. This is accomplished by a mechanism that is called 'leaky ribosomal scanning', in which the translation machinery occasionally slips past the first start codon and translation can initiate at later start codons. In the case of the βA3/A1 messenger, there are two start codons that lie in the same reading frame, leading to the expression of two essentially identical proteins that only differ in the length of their N-terminal extension. It was unclear, however, whether the short length of the 5' UTR of the βA3/A1 messenger (five nucleotides in most species known today [Goring & Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Peterson & Piatigorsky, 1986]) was responsible for this phenomenon, or whether it was caused by the fact that the first start codon on this messenger can be classified as 'weak'

according to the definition of Kozak (1989). Chapter IV clearly confirms that βA3- and βA1-crystallin can indeed be produced from the single βA3/A1 messenger. Moreover, extending the length of the 5' UTR of this messenger to well over 60 nucleotides leads to the expression of just βA3, indicating that the short length of the 5' UTR of the wild-type messenger and not the weak context of the first start codon is the determining factor for the occurrence of leaky ribosomal scanning. This is confirmed by the fact that in chicken the first start codon is 'strong', but both βA3- and βA1-crystallin proteins can still be detected in chicken eye lenses.

It is very difficult to speculate about the history of the βA3/A1 gene, although the fact that several β-crystallins possess a non-coding first exon [Wistow, 1995] may indicate that βA1 existed as the single product of the ancestral βA3/A1 gene before the βA3 start codon was introduced by a chance mutation in the non-coding first exon of this gene. Alternatively, however, all other β-crystallins but βA3/A1 -- and βB1 too, for that matter -- may just have lost expression of the first exon, and the occurrence of leaky scanning of the βA3/A1 mRNA may thus equally well be explained by shortening of the 5' UTR during the course of evolution. Since it is impossible to resolve this point, it is more important to focus on the fact that this mechanism, once it was introduced, has been conserved for at least 350 million years during evolution, since it can be found in species ranging from birds to mammals [Goring & Horwitz, 1984; Quax-Jeuken *et al.*, 1984; Hogg *et al.*, 1986; Peterson & Piatigorsky, 1986], and possibly even in frogs [Lu *et al.*, 1996]. Clearly, therefore, the expression of both βA3 and βA1 from this messenger via leaky ribosomal scanning was beneficial to the lens.

The fact that the lengths of the N-terminal extensions of βA3 and βA1 -- 30 and 17 aminoacid residues respectively -- have been perfectly conserved during evolution and neither βA3 nor βA1 has been sacrificed to improve expression of the other, plus the fact that leaky ribosomal scanning -- although fairly common for viral genes -- is very rare for eukaryotic genes and always has implications for the proteins involved [Shaper *et al.*, 1988; Tuboi *et al.*, 1990; Descombes & Schibler, 1991; Slusher *et al.*, 1991; Pietrini *et al.*, 1992; Lin *et al.*, 1993; Ossipow *et al.*, 1993; Tenhunen & Ulmanen, 1993; Spotts *et al.*, 1997], obviously indicates that both proteins are required in balancing amounts to fulfill some function in the lens. The only question that remains to be answered then is: "What is this function?"

The answer to this question must lie in the N-terminal extensions of βA3- and βA1-crystallin, since this is the only difference between them. The average extension of the β-crystallins has a length of about 15 residues, which is not too far from the 13 residues of the βA1 N-terminal extension. There are two β-crystallins that have clearly longer extension:

βA3, which has N-terminal extension of 30 residues, and βB1, which has an N-terminal extension that can be as long as 58 residues [Quax-Jeuken *et al.*, 1984]. βB1-Crystallin has been found to be associated with the higher molecular weight fraction, βH [Berbers, 1982]. It was therefore suggested early on [Berbers *et al.*, 1983; Slingsby, 1985] that the β-crystallin extensions were involved in protein-protein interactions that would explain their oligomerization, in contrast to the extension-less γ-crystallins that do not oligomerize. Several truncations were made to test this hypothesis, but different results pointed in different directions and no real consensus was reached [Hope *et al.*, 1994; Kroone *et al.*, 1994; Trinkl *et al.*, 1994]. Chapter II clearly shows that the N-terminal extension of βA3 is solvent exposed and flexible -- as shown by ^{1}H-NMR -- and that there is no difference in oligomerization behavior of βA3 and βA1, since both βA3 and βA1 form homo-dimers *in vitro* and can be found in similar ratios in the different size classes of eye lens β-crystallins when separated by gel permeation chromatography. It is thus concluded from this Chapter that the N-terminal extensions of βA3 and βA1 are not involved in protein-protein interactions, neither in dimer formation nor in formation of mixed oligomers. Consistent with these conclusions, Trinkl *et al.* (1994) have shown that the difference in sequence and conformation of the connecting peptide of the β- and γ-crystallins is most likely responsible for dimerization of the β-crystallins and the monomeric state of the γ-crystallins, as suggested previously by others [Bax *et al.*, 1990; Lapato *et al.*, 1991]. In Chapter II, another difference between βA3 and βA1 is noted, namely the strong difference in charge and pI of their N-terminal extensions, which is true for all species known today in spite of differences in protein sequences between species. Since the N-terminal extensions of these proteins are not involved in direct protein-protein interactions, it is suggested that these charges could be involved in the non-specific, short-range protein interactions that are so important in determining the physical properties of the eye lens (see Chapter I). The negative charges on the βA3 N-terminal extension lead to repulsive interactions between the individual βA3 dimers, thus avoiding aggregation and favoring transparency. The absence of these charges in the N-terminal extension of βA1 causes reduced repulsion. In the mixed β-crystallin complexes of the eye lens, this would mean that complexes containing βA3 would exhibit more repulsive interactions, thereby favoring lens transparency, whereas complexes containing βA1 would be less repulsive, thereby allowing closer packing and higher concentrations of proteins in the eye lens [Tardieu *et al.*, 1992].

This idea has been further extended in Chapter III, where the truncation of βA3- and βA1-crystallin N-terminal extensions was studied in the bovine eye lens. It was found that βA3/A1 truncation products could already be detected in 0.4 year-old bovine eye lenses. The two main products were identified by protein sequencing to correspond to βA3 missing 11 or 22 residues from its N-terminus, the latter of which could also be formed by removing 4 residues from the βA1 N-terminal extension. Given the fact that βA3(-22) can be found in cortex and nucleus, whereas βA3(-11) can only be found in nucleus, it was concluded that two proteolytic enzymes must be involved. This is consistent with observations by Shih *et al.* (1998), who have shown that calpain -- a protein held responsible for numerous protein cleavages in the eye lens [David *et al.*, 1994] -- is able to produce βA3(-11), but not βA3(-22) when added to fetal calf lens proteins *in vitro*. The fact that, upon aging, βA3 disappears faster than βA3(-11) and βA1, leads to the conclusion that the longer the N-terminal extension (βA3 > βA3(-11) > βA1) the more vulnerable these proteins are for truncation at position 22.

In addition to cleavage products of βA3/A1, numerous charge variants -- possibly caused by deamidations -- start to accumulate upon aging, notably in the water-insoluble fractions of the bovine eye lens. The fact that some of these charge variants are almost exclusively found in the water-insoluble fractions, while the direct truncation products of βA3/A1 are not over-represented in the insoluble fractions, led to the conclusion that protein modifications other than truncation might be the major cause of insolubilization of β-crystallin proteins and that truncation of βA3 and βA1 may actually be of functional importance to the eye lens. In the cortex, were protein synthesis still takes place, βA3 crystallin levels remain high. Towards the nucleus, however, βA3 is rapidly truncated during aging. Given the fact that the β-crystallin N-terminal extension may play an important role as physical spacers [Trinkl *et al.*, 1994] or as carriers of repulsive charge that regulate the short-range, non-specific protein interaction in the eye lens [Werten *et al.*, 1996], truncation would lead to loss of spacers and/or repulsive charges in βA3 containing complexes. This would then result in closer packing in areas where this truncation occurs. Truncation of βA3 has been shown in Chapter III to gradually increase from cortex to nucleus, coinciding with the necessity for tighter protein packing towards the center of the lens, thus helping the eye lens to maintain the protein concentration gradient that is so crucial for its correct refractive properties.

This role of the N-terminal extensions of βA3 and βA1 in short-range, non-specific protein interactions, and their modulation via protein cleavage during normal aging of the eye lens, may very well be the answer to the question raised earlier in this Chapter, "What is this function" of βA3 and βA1?

Although it has become very clear that, at least for βA3 and βA1, the sequence extensions are not used for specific protein-protein interactions, this does not appear to be true for all β-crystallins in general. Chapter IV describes the spontaneous formation of βA3/βB2 mixed complexes that are dimeric at low (< 1 mg/ml), but tetrameric at higher protein concentrations. ^{1}H-NMR spectroscopy at high protein concentration has shown that the N-terminal extensions of βA3 remain solvent exposed in the βA3/βB2 hetero-tetramer, whereas resonances from both the N- and C-terminal βB2 extensions -- that are flexible in the homo-dimer [Carver, 1993] -- disappear from the spectrum. It is concluded that both of the βB2 extensions are involved in protein-protein interactions in the mixed tetramer and thus may help stabilize and perhaps even promote the formation of this particular complex, which is one of the major components of the tetrameric β-crystallin fraction of the eye lens [Slingsby & Bateman, 1990]. In the hetero-dimer, only the C-terminal extension of βB2 may be involved in such interactions, but this is solely based on models and could not be verified experimentally. The fact that the function of the β-crystallin extensions can evidently differ for the various β-crystallin subunits, and may even differ for particular complexes (see Chapter IV), again demonstrates another level of variation that was introduced into the crystallins during evolution of the eye lens.

7.3 Iota-crystallin

As noted before, taxon-specific crystallins may have been used during evolution as a way to further enhance or even extend functions of the eye lens in specific species. In the case of diurnal geckos, which probably evolved from diurnal lizards that first became nocturnal and then reverted to diurnal lifestyle again [Walls, 1942], it is no wonder that these dramatic changes have led to the recruitment of several taxon-specific crystallins [Röll *et al.*, 1995; Jimenez-Asensio *et al.*, 1995; Röll *et al.*, 1996]. Certainly the most interesting of these proteins is iota-crystallin, since its properties relate directly to its function in the gecko eye lens. Its recruitment is another example of molecular mechanisms that have proven successful during the evolution of the eye lens in this species.

Iota-crystallin was first discovered in the eye lenses of the diurnal gecko *Lygodactylus picturatus* [Röll *et al.*, 1996]. Through protein sequencing it was found to be closely related to rat cellular retinol-binding protein type I (CRBP I). In contrast to CRBP I, which has all-*trans*-retinol as its natural ligand, iota-crystallin was found to contain all-*trans*-3,4-didehydroretinol, also known as vitamin A_2. The vitamin A_2 · iota-crystallin complex gives the eye lenses of this gecko a dark yellow color that improves the optical quality of these lenses and, more importantly, functions as a filter for short-wave UV radiation. Because the eyelids in these animals are fused to form a transparent spectacle and because geckos cannot regulate the aperture of their iris, they cannot control the amount of light that enters their eyes. Since these animals live in the savanna areas of South-East Africa and are thus exposed to high light intensities, the presence of such an effective UV filter would certainly be beneficial.

Because only the partial protein sequence of iota-crystallin was known [Röll *et al.*, 1996], and no information was available on the gecko CRBP I protein, it was unclear whether iota-crystallin represents a gene duplication product of gecko CRBP I that was optimized to function as a lens protein, or whether iota-crystallin is in fact the gecko CRBP I that has been upregulated in the lens to function as a crystallin. In Chapter VI, the cloning of iota-crystallin from *Lygodactylus* eye lenses and the cloning of CRBP I from *Lygodactylus* liver is described. Since the two cDNA sequences were identical, even in the 3' UTRs, and no further CRBP I-like sequences were found, it was concluded that iota-crystallin really is gecko CRBP I, upregulated in the gecko eye lens. The complete sequence of iota-crystallin/CRBP I was used to build a model from the known rat CRBP I 3D-structure. A general rigidification of the protein compared to rat CRBP I seems to have optimized iota-crystallin for its function as a stable lens protein. The observed decrease in negative surface charges may enable the protein to achieve the close packing and high protein concentration that exists in the eye lens. Some mutations in the binding pocket of iota-crystallin could either be tentatively interpreted as being adaptations for preferential binding of vitamin A_2 or equally well as selectively neutral changes. Therefore, iota-crystallin was expressed as a recombinant protein and purified to study its actual ligand-binding properties. Chapter VI shows that iota-crystallin is able to bind both retinol and vitamin A_2 with similar and high affinity (K_d ~ 1.6 nM), as is the case for other CRBP I proteins [Malpeli *et al.*, 1995]. This means that no real optimization of the iota-crystallin/CRBP I protein was required to make this protein fulfill its new role in the gecko eye lens. However, the most difficult part in the recruitment of CRBP I/iota-crystallin as a lens protein, and in fact the only way that this recruitment would make any sense, was to simultaneously get vitamin A_2 into the lens. Retinol, the natural ligand of CRBP I, would not

have been an alternative to vitamin A_2, since retinol bound to CRBP I fluoresces in the range of visible light and, furthermore, its binding to CRBP I is photosensitive. Since this is not the case for vitamin A_2, only this retinoid could be used as a lens chromophore. Since vitamin A_2, in contrast to retinol, is not normally present in gecko ocular tissues, but can be found in the retina of diurnal geckos that possess iota-crystallin [Röll & Schwemer, 1999], this must have involved the activation of an enzyme in the gecko retina that converts retinol to vitamin A_2 specifically for this purpose, plus the generation of an as of yet unknown vitamin A_2 transport system from the gecko retina to the lens.

In conclusion this shows that molecular evolution was able to create a totally new function for the gecko eye lens as a UV filter to protect the gecko retina from UV damage, by cleverly bringing together two existing molecular building blocks to perform a new task. This certainly is a beautiful example of the efficiency, ingenuity and success of evolutionary strategies.

7.4 Epilogue

The eye lens can indeed be considered a true playground for evolution, where it had the opportunity to experiment with all kinds of molecular mechanisms. Although the nature of the eye lens must have restricted evolution in the sense that any mistake could lead to vision impairment and thus to negative selection, it allowed for great diversity given the enormous differences in requirements for eye lenses of organisms living in vastly different environments. The fact that during some stages of evolution quick adaptations were necessary no doubt contributes to the overall diversity of the crystallins we see today and of the molecular mechanisms that were used for their recruitment and optimization as lens proteins. These mechanisms, as found in the eye lens, have no doubt occurred in the evolution of other tissues as well. The eye lens therefore is a very useful model system to study fundamental molecular mechanisms and strategies of evolution.

References

Bax B, Lapatto R, Nalini V, Driessen H, Lindley PF, Mahadevan D, Blundell TL & Slingsby C (1990). *Nature*, **347**, 776-780.

Berbers GA, Boerman OC, Bloemendal H & de Jong WW (1982). *Eur. J. Biochem.* **128**, 495-502.

Berbers GA, Brans AM, Hoekman WA, Slingsby C, Bloemendal H & de Jong WW (1983). *Biochim. Biophys. Acta* **748**, 213-219.

Carver JA, Cooper PG & Truscott RJ (1993). *Eur. J. Biochem.* **213**, 313-320.

Cvekl A & Piatigorsky J (1996). *Bioessays* **18**, 621-630.

David LL, Azuma M & Shearer TR (1994). *Invest. Ophthalm. Vis. Sci.* **35**, 785-793.

Descombes P & Schibler U (1991). *Cell* **67**, 569-579.

Gorin MB & Horwitz J (1984). *Curr. Eye Res.* **3**, 939-948.

Hogg D, Tsui LC, Gorin M & Breitman ML (1986). *J. Biol. Chem.* **261**, 12420-12427.

Hope JN, Chen HC & Hejtmancik JF (1994). *Protein Eng.* **7**, 445-451.

Jimenez-Asensio J, Gonzalez P, Zigler Jr. JS & Garland DL (1995). *Biochem. Biophys. Res. Comm.* **209**, 796-802.

Kozak M (1989). *J. Cell Biol.* **108**, 229-241.

Kroone RC, Elliott GS, Ferszt A, Slingsby C, Lubsen NH & Schoenmakers JGG (1994). *Protein Eng.* **7**, 1395-1399.

Lapatto R, Nalini V, Bax B, Driessen H, Lindley PF, Blundell TL & Slingsby C (1991). *J. Mol. Biol.* **222**, 1067-1083.

Lin FT, MacDougald OA, Diehl AM & Lane MD (1993). *Proc. Natl. Acad. Sci. USA* **90**, 9606-9610.

Lu SF, Pan FM & Chiou SH (1996). *Biochem. Biophys. Res. Commun.* **221**, 219-228.

Lubsen NH, Aarts HJM & Schoenmakers JGG (1988). *Prog. Biophys. Mol. Biol.* **51**, 47-76.

Malpeli G, Stoppini M, Zapponi MC, Folli C & Berni R (1995). *Eur. J. Biochem.* **229**, 486-493.

Ossipow V, Descombes P & Schibler U (1993). *Proc. Natl. Acad. Sci. USA* **90**, 8219-8223.

Peterson CA & Piatigorsky J (1986). *Gene* **45**, 139-147.

Piatigorsky J & Wistow GJ (1989). *Cell*, **57**, 197-199.

Pietrini G, Aggujaro D, Carrera P, Malyszko J, Vitale A & Borgese N (1992). *J. Cell Biol.* **117**, 975-986.

Quax-Jeuken Y, Janssen C, Quax W, van den Heuvel R & Bloemendal H (1984). *J. Mol. Biol.* **180**, 457-472

Röll B, van Boekel MA, Amons R & de Jong WW (1995). *Biochem. Biophys. Res. Comm.* **217**, 452-458.

Röll B, Amons R & de Jong WW (1996). *J. Biol. Chem.* **271**, 10437-10440.

Röll B & Schwemer J (1999). *J. Comp. Physiol. A* (in press)

Shaper NL, Hollis GF, Douglas JG, Kirsch IR & Shaper JH (1988). *J. Biol. Chem.* **263**, 10420-10428.

Shih M, Lampi KJ, Shearer TR & David LL (1998). *Mol. Vis.* **4**, 4.

Slingsby C (1985). *Trends Biochem. Sci.* **10**, 281-284.

Slingsby C & Bateman OA (1990). *Biochemistry* **28**, 6593-6599.

Slusher LB, Gillman EC, Martin NC & Hopper AK (1991). *Proc. Natl. Acad. Sci. USA* **88**, 9789-9793.

Spotts GD, Patel SV, Xiao Q & Hann SR (1997). *Mol. Cell. Biol.* **17**, 1459-1468.

Tardieu A, Veretout F, Krop B & Slingsby C (1992). *Eur. Biophys. J.* **21**, 1-12.

Tenhunen J & Ulmanen I (1993). *Biochem. J.* **296**, 595-600.

Trinkl S, Glockshuber R & Jaenicke R (1994). *Protein Sci*, **3**, 1392-1400.

Tuboi S, Suzuki T, Sato M & Yoshida T (1990). *Adv. Enzyme Regul.* **30**, 289-304.

Walls GL (1942). *The vertebrate eye and its adaptive radiation*, pp. 622-640, Cranbrook Institute of Science, Bloomfield Hills, MI.

Wistow GJ (1995). *Molecular biology and evolution of crystallins: gene recruitment and multifunctional proteins in the eye lens*, R.G. Landes Company, Austin, TX.

Summary

From the dawn of evolution where primitive one-cellular organisms oriented themselves to the light of our sun by simple photoreceptors in search of energy to sustain their existence, to the current day where light from the environment is captured by our eyes, focused on the retina by the eye lens and then processed by our brain to useful visual information, light has been one to the strongest driving forces ever to have determined the course of evolution. Although eyes have been invented multiple times during evolution, and the great diversity of eyes found in species today represents the various solutions to a similar problem, very ancient underlying molecular mechanisms have been maintained in all species and still guide the development of eyes at various levels.

The vertebrate eye is one of the more advanced types of eyes that can be found in animals today. It is characteristically called a 'camera-type' eye. The central position in the vertebrate eye is taken by the eye lens. This remarkable tissue has two properties crucial to vision. First, it is transparent, allowing light to pass through. Second, it is able to refract light, allowing the correct focusing of light on the retina. Responsible for both of these properties are the eye lens crystallins, structural proteins that are present in high concentrations in the lens fiber cells. The crystallins are stable and highly water-soluble proteins that have to last a lifetime withstanding the deleterious effects of sunlight. It makes sense, therefore, that crystallins in general seem to have been recruited from proteins that have some kind of protective properties. This is true for the ubiquitous α-, β- and γ-crystallins that can be found in varying amounts in all vertebrates, as well as for the various taxon-specific proteins that are found only in certain evolutionary lineages.

The diversity of the current-day crystallins bears evidence to the various strategies and molecular mechanisms that have been used by evolution to perfect the lens. Examples of such mechanisms are gene sharing, gene duplication, and gene fusion that all have played important roles in the recruitment and diversification of the α-, β- and γ-crystallins, as well as of some of the taxon-specific crystallins. These mechanisms that have been applied so successfully in the lens, represent paths that are often followed by evolution in general. Two somewhat unusual examples of these molecular mechanisms and particularly the properties of the proteins involved have been described in detail in this thesis. The first is the production of βA3- and βA1-crystallin from the single βA3/A1 mRNA through 'leaky ribosomal scanning' (see Chapter IV), leading to two almost identical proteins that only differ in the length and charge of their N-terminal extensions. It has been shown that these extensions are not

involved in oligomerization of the β-crystallins, but rather serve to modulate the short-range, non-specific protein interactions that are crucial for transparency and proper refractive index of the lens (Chapter II). The gradual proteolytic cleavage of the βA3 N-terminal extension towards the center of the lens seems to be an ingenious way to help establish the protein concentration gradient that is necessary for optical quality of the eye lens (Chapter III). Thus, the interplay of both βA3 and βA1 is required for proper lens functioning. It was also shown that the extensions of other β-crystallins may serve different purposes, such as the N- and C-terminal extensions of βB2, that seem to specifically promote formation of the βA3/βB2-crystallin hetero-tetramer via protein-protein interactions (Chapter V). These different roles for similar structural elements add to the overall complexity and diversity of the crystallin components of the lens.

The second example of unusual molecular mechanisms applied by evolution is the unique combination of iota-crystallin and vitamin A_2 in the eye lenses of some diurnal geckos (Chapter VI). The iota-crystallin · vitamin A_2 complex functions as a UV-filter in these animals. Vitamin A_2, which is not normally present in gecko eye tissues, can be found in the retina of diurnal geckos that posses iota-crystallin. This means that, besides expression of CRBP I in the lens, activation of an enzyme converting retinol to vitamin A_2 in the gecko retina plus development of an as of yet unknown system to transport this retinoid from the retina to the lens were required. Only the simultaneous occurrence of all of these events could have led to the beneficial effect and positive selective forces necessary to maintain the new crystallin. This unusual combination shows that evolution is able to bring together existing molecular building blocks and to combine them in such a way that new functions can be achieved.

Studying the mechanisms that led to the recruitment and diversification of the eye lens crystallins thus gives us the opportunity to better understand the molecular biology of the fundamental strategies of evolution. Studying the specific and non-specific interactions that exist between the various crystallins in the eye lens can greatly enhance our general understanding of structure/function relationships in proteins. As such, the eye lens can be a useful model system for various disciplines of modern day science.

Samenvatting

Vanaf het prilste begin van de evolutie toen primitieve eencellige organismen zich door middel van simpele fotoreceptoren tot onze zon richtten, op zoek naar de energie die zo belangrijk was voor hun bestaan, tot op de dag van vandaag waar licht vanuit onze omgeving wordt opgevangen door onze ogen, door de ooglens correct op de retina wordt gefocusseerd, en vervolgens door onze hersenen wordt omgezet in bruikbare visuele informatie, is licht een van de sterkste invloeden geweest die ooit de loop van de evolutie hebben bepaald. Hoewel ogen vele malen onafhankelijk van elkaar tijdens de evolutie zijn ontstaan, en de grote diversiteit aan ogen zoals die in de hedendaagse diersoorten wordt aangetroffen verschillende oplossingen voor eenzelfde probleem vormen, blijken zeer oude achterliggende moleculaire mechanismen in al deze dieren bewaard te zijn gebleven en nog steeds op diverse niveaus de ontwikkeling van ogen te sturen.

Het oog van de vertebraten is één van de meer geavanceerde ogen die men in het dierenrijk tegenkomt. Vanwege zijn karakteristieke structuur kan dit type oog gezien worden als een soort camera. Een centrale positie wordt daarbij ingenomen door de ooglens. Dit bijzondere weefsel heeft twee eigenschappen die cruciaal zijn voor het gezichtsvermogen. Allereerst is de ooglens transparant, waardoor licht doorgelaten kan worden. Verder kan de lens licht breken, waardoor het correct op het netvlies gefocusseerd kan worden. Verantwoordelijk voor beide eigenschappen zijn de structurele ooglenseiwitten, de zogenaamde crystallines, die in hoge concentraties in de lensvezelcellen voorkomen. Crystallines zijn zeer stabiele en wateroplosbare eiwitten die een leven lang de schadelijke gevolgen van zonlicht moeten weerstaan. Het is daarom wellicht niet verwonderlijk dat de crystallines in het algemeen geselecteerd lijken te zijn uit eiwitten die een of andere beschermende eigenschap hebben. Dit is niet alleen het geval voor alom vertegenwoordigde α-, β- en γ-crystallines, maar ook voor de taxon-specifieke crystallines die slechts in bepaalde evolutionaire lijnen voorkomen.

De diversiteit aan hedendaagse crystallines toont de verschillende strategieën en moleculaire mechanismen die tijdens de evolutie een rol gespeeld hebben om de ooglens te perfectioneren. Voorbeelden van zulke mechanismen zijn gen-duplicatie, gen-fusie en het toekennen van meerdere functies aan één gen-produkt. Al deze mechanismen hebben een belangrijke rol gespeeld in de acquisitie en diversificatie van de α-, β- en γ-crystallines, maar ook van sommige taxon-specifieke crystallines. Deze mechanismen, die zo succesvol in de lens zijn toegepast, hebben ook hun relevantie buiten de lens als zijnde algemeen geldende evolutionaire strategieën. Twee van de meer uitzonderlijke voorbeelden van deze strategieën, en met name ook de eigenschappen van de betrokken eiwitten, zijn in dit proefschrift in detail beschreven. Het eerste voorbeeld betreft de expressie van βA3- en βA1-crystalline vanaf één

enkel mRNA, via een mechanisme dat 'leaky ribosomal scanning' wordt genoemd (zie Hoofdstuk IV), waarbij twee bijna identieke eiwitprodukten ontstaan die slechts verschillen in hun N-terminale extensies. Er is aangetoond dat deze extensies niet betrokken zijn bij de oligomerisatie van de β-crystallines, maar bijdragen aan het reguleren van de niet-specifieke lokale interacties die zo belangrijk zijn voor de transparantie en de juiste brekingsindex van de lens (Hoofdstuk II). Vooral de geleidelijke klieving van de N-terminale extensie van βA3 lijkt een ingenieuze manier om bij te dragen aan de eiwit-concentratie gradiënt die noodzakelijk is voor een goede optische kwaliteit van de lens (Hoofdstuk III). Een samenspel van βA3 en βA1 lijkt dus noodzakelijk voor het goed functioneren van de lens. Verder is aangetoond dat de extensies van andere β-crystallines andere functies kunnen hebben, zoals bijvoorbeeld de N- en C-terminale extensies van βB2, die betrokken zijn in eiwit/eiwit-interacties en zo specifiek de vorming van het βA3/βB2-crystalline hetero-tetrameer lijken te bevorderen (Hoofdstuk V). Deze verschillende functies van vergelijkbare structurele elementen dragen bij aan de totale complexiteit en diversiteit van de crystallines in de lens.

Het tweede voorbeeld van ongebruikelijke moleculaire mechanismen die tijdens de evolutie van de lens opgetreden zijn, is de combinatie van iota-crystalline en vitamine A_2 in de lenzen van sommige dag-actieve gekko's (Hoofdstuk VI). Het complex van iota-crystalline en vitamine A_2 functioneert als een UV-filter bij deze dieren. Vitamine A_2 wordt normaal gesproken niet in de ogen van gekko's aangetroffen, maar is daarentegen wel aangetoond in de retina van deze dag-actieve gekko's die iota-crystalline bezitten. Dit betekent dat er naast de expressie van CRBP I in de lens ook een enzym in de gekko retina geactiveerd moet zijn dat retinol in vitamine A_2 omzet, en dat er daarnaast een vooralsnog onbekend transport-mechanisme ontwikkeld moet zijn dat dit vitamine A_2 vanuit de retina naar de lens transporteert. Alleen het gelijktijdig optreden van al deze gebeurtenissen kan een gunstig effect hebben gehad en voor de positieve selectie hebben gezorgd die dit crystalline heeft doen behouden. Dit ongebruikelijke voorbeeld toont aan dat de evolutie in staat is bestaande moleculaire bouwstenen bij elkaar te brengen en deze zodanig te combineren dat nieuwe functies kunnen ontstaan.

Het bestuderen van de mechanismen die hebben geleid tot de acquisitie en diversificatie van de crystallines in de ooglens stelt ons in staat om de moleculaire biologie en de fundamentele strategieën van de evolutie beter te begrijpen. Daarnaast kan het bestuderen van de specifieke en niet-specifieke interacties die tussen de verschillende crystallines onderling bestaan ons inzicht in de structuur/functie-relaties van eiwitten in het algemeen sterk vergroten. Als zodanig is de lens een zeer nuttig modelsysteem voor diverse takken van de hedendaagse wetenschap.

Dankwoord

Het is al vaker gezegd en het is inderdaad zo: promoveren doe je niet alleen. Ik wil daarom de volgende mensen speciaal bedanken, die elk op hun eigen wijze hebben bijgedragen aan een succesvol verlopen promotietijd:

Ole en Marjon, als de eerste medebewoners van Lab. 1.59 hebben we met z'n drieën een grote lege ruimte toch gezellig kunnen maken! Ook kon ik bij jullie altijd terecht als mijn vraagbaak voor Moleculair Biologische en Fylogenetische vraagstukken. Ronald en Bas, als dynamisch duo op eiwit-gebied waren jullie goede collega's in het expressie- en zuiveringswerk. Guido, als computer-nerds onder elkaar hebben we zeker de nodige Gigabytes aan informatie uitgewisseld! Perry en Tinie, als HPLC-experts en mede-Canon-eigenaren kon ik altijd bij jullie terecht voor vragen over de apparatuur en gesprekken over fotografie. Al mijn andere collega's ben ik zeer dankbaar voor de plezierige werksfeer waarin ik met hen heb mogen samenwerken.

Sehr herzlich möchte ich auch Herrn Prof. Dr. Rainer Jaenicke danken, der mir die möglichkeit geboten hat in Regensburg Experimente aus zu führen um die Stabilität von βA3 und βA1 zu vergleichen, und der außerdem die Analysen mit der Ultracentrifuge für mich ausgeführt hat. Stefan Palme, Eva-Maria Mayr und Karin Berr danke ich für ihre Hilfe bei den Experimenten und für eine schöne Zeit in Regensburg.

I would also like to thank Dr. Christine Slingsby for sharing her thoughts and vast knowledge on protein structure with me.

Wilfried, jou wil ik in het bijzonder bedanken voor de goede begeleiding die je me gegeven hebt -- waarbij je me altijd vrij gelaten hebt om zelfstandig mijn weg te zoeken -- en voor de subtiele manier waarmee je me de fijne kneepjes van het schrijven van artikelen hebt bijgebracht. Er komt best wel iets bij kijken om een verhaal goed en helder over te brengen, en om een tekst op te bouwen vanuit lezersperspectief. Bedankt hiervoor.

Als laatste wil ik jou bedanken, Anita, want met name in deze laatste periode -- bij het schrijven van dit proefschrift -- heb jij ongelofelijk veel geduld met mij gehad! Hele weekenden en avonden achter de computer, met maar weinig tijd om samen iets leuks te doen: dat zijn dingen waar je als partner van een promovendus toch maar even mee om moet kunnen gaan. Desalniettemin ben jij tijdens mijn gehele promotietijd m'n lieve steun en toeverlaat geweest. Bedankt voor je liefde en al de keren dat je mij hebt weten te motiveren... Ik hou van je!

Resume

Paul J.L. Werten was born on July 10, 1969 in Geffen, the Netherlands. He studied Chemistry at the University of Nijmegen, Nijmegen, the Netherlands from 1987 until 1993, and graduated with a major emphasis in Biochemistry and a minor emphasis in Clinical Chemistry. He was a PhD student at the laboratory of Wilfried W. de Jong, PhD, from 1993 until 1998, where he carried out the research described in this thesis. He attended the ARVO Meeting in Fort Lauderdale, Florida, USA in 1996 and in 1997, and was invited speaker at the XIII ICER Meeting in Paris, France, in 1998, for which he received an ISER Travel Fellowship. In the same year he joined the Cell Physiology department led by Carel van Os, PhD, and Peter Deen, PhD, and as a post-doc he is currently involved in a EU Biotech project dealing with the structure, function, regulation and exploitation of various members of the MIP family of water and glycerol transporters.

Curriculum Vitae

Paul J.L. Werten is op 10 Juli 1969 geboren in Geffen. Hij bezocht het Maasland-College te Oss, waar hij in 1987 zijn Atheneum-B diploma behaalde. Hij studeerde van 1987 tot 1993 Scheikunde aan de Katholieke Univerisiteit Nijmegen, en rondde zijn studie af met een uitgebreid hoofdvak Biochemie en een bijvak Klinische Chemie. Hij was van 1993 tot 1998 onder supervisie van Prof. Dr. Wilfried W. de Jong werkzaam als Assistent in Opleiding bij de vakgroep Biochemie van de Faculteit der Natuurwetenschappen, alwaar hij het onderzoek uitvoerde dat in dit proefschrift beschreven is. Hij nam in 1996 en 1997 deel aan de ARVO Bijeenkomst in Fort Lauderdale, Florida, Verenigde Staten, en was in 1998 uitgenodigd als spreker bij de 13de ICER Bijeenkomst in Parijs, Frankrijk, waarvoor hij een ISER Reisbeurs ontving. In ditzelfde jaar begon hij als post-doc bij de vakgroep Celfysiologie onder leiding van Dr. Carel van Os en Dr. Peter Deen aan een EU Biotech project dat zich bezighoudt met de structuur, functie, regulatie en exploitatie van de MIP eiwitfamilie van water- en glycerol-kanalen.

Publications

Prinsen CF, Werten PJL, Maassen JA & Veerkamp JH (1994). "No significant tyrosine phosphorylation of muscle fatty acid-binding protein". *Biochim. Biophys. Acta* **1215**, 103-108.

Werten PJL, Carver JA, Jaenicke R & de Jong WW (1996). "The elusive role of the N-terminal extension of βA3- and βA1-crystallin". *Protein Eng.* **9**, 1021-1028.

Werten PJL, Vos E & de Jong WW (1999). "Truncation of βA3/A1-crystallin during aging of the bovine lens: possible implications for lens optical quality". *Exp. Eye Res.* **68**, 99-103.

Werten PJL, Stege GJJ & de Jong WW (1999). "The short 5' untranslated region of the βA3/A1 mRNA is responsible for leaky ribosomal scanning". *Mol. Biol Rep.* **26**, 201-205.

Werten PJL, Lindner RA, Carver JA & de Jong WW (1999). "Formation of βA3/βB2-crystallin mixed complexes: involvement of N- and C-terminal extensions". *Biochim. Biophys. Acta* **1432**, 286-292.

Werten PJL, Röll B, van Aalten DMF & de Jong WW (1999). "Gecko iota-crystallin: how cellular retinol-binding protein became an eye lens ultraviolet filter". *Accepted for publication in Proc. Natl. Acad. Sci. USA*

Published Abstracts

Werten PJL, Jaenicke R & de Jong WW (1996). "The association behavior of βA3- and βA1-crystallin". *Invest. Ophtalmol. Vis. Sci. (Abstract Book)*, S597. ARVO Meeting, Fort Lauderdale, Florida, April 21-26, 1996.

Werten PJL, Vos E & de Jong WW (1997). "Proteolysis of βA3/A1-crystallin during normal aging of the bovine lens". *Invest. Ophtalmol. Vis. Sci. (Abstract Book),* S297. ARVO Meeting, Fort Lauderdale, Florida, May 11-16, 1997.

Werten PJL, Röll B & de Jong WW (1998). "Iota-crystallin: the gecko's sunglasses". *Exp. Eye Res. (Abstract Book)*, S17. The XIII ICER Meeting, Paris, France, July 26-31, 1998.

Fellowships

ISER Travel Fellowship to attend the XIII ICER Meeting, Paris, France, July 26-31, 1998. Invited speaker at this meeting.

EMBO Short Term Fellowship to conduct research at the Laboratory of Dr. Andreas Engel, Biocenter of the University of Basel, Basel, Switzerland, January 30-February 12, 2000.